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Effects Of Cycloheximide On Polyoma Dna Replication

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EFFECTS OF CYCLOHEXIMIDE ON POLYOMA DNA REPLICATION

BY

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ABSTRACT

As a direct result of treatment with 10 µg/ml of cycloheximide (CX) in polyoma-infected secondary mouse embryo tissue cultures, a dramatic quantitative reduction of polyoma (Py) form I DNA synthesis was observed. The present study revealed a comparable reduction in the size of the Py replicative intermediate (RI) pool while maintaining a turnover rate equivalent to that of an untreated control. Both the rate of the elongation of nascent fragments into progeny strands and the rate of RI maturation into closed-circular DNA are unaffected. This leads to the conclusion that the observed inhibition is exclusively at the level of initiation of new rounds of genome replication.

DNA generated subsequent to CX treatment showed no marked deviation from the normal base composition, molecular weight, or population uniformity. A notable qualitative alteration expressed itself as a change in superhelicity of progeny molecules, equivalent to $\Delta\sigma_o = 0.0195$. This DNA, termed form Ic, replicates on a form I DNA template via an apparently normal RI, i.e. the RI population retains its characteristic 25 S sedimentation property under neutral conditions and

possesses the typical banding position in a propidium diiodide-cesium chloride isopycnic analysis. Limited recycling was observed after CX treatment; only 35% of the radioactivity from two hour pre-labeled form I DNA was subsequently recovered in the form Ic population. The synthesis of Ic is dependent upon on-going DNA replication. The rate of both amino acid and thymidine incorporation returned to normal efficiently and completely following the release of the CX block. Under these conditions, approximately two-thirds of the Ic population regains the superhelix density of form I. This recovery is independent of DNA replication.

Kinetic data indicate that the factor(s) maintaining proper superhelix density of newly-synthesized polyoma DNA is distinct from those which control the initiation of new rounds of genome replication.

Last, but not least, I thank my wife, Carolyn, for her unwaivering support, patience, and understanding throughout the course of this work.

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To Carolyn, my wife

LIST OF ABBREVIATIONS

A	absorbance
aa	amino acids
ara-C	cytosine arabinoside
BND-cellulose	benzoylated-naphthoylated DEAE cellulose
^{14}C-marker	carbon 14-labeled marker
^{14}C-TdR	2-^{14}C-thymidine
CPM	counts per minute
CX	cycloheximide
Ci	currie
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
FCS	foetal calf serum
FUdR	5-fluorodeoxyuridine
^{3}H-TdR	[methyl-^{3}H] thymidine
HU	hydroxyurea
ICNV	International Committee on Nomenclature of Viruses now known as: International Committee on Taxonomy of Viruses (ICTV)
M	mole
METC	mouse embryo tissue culture
µCi	micro-currie
m.o.i.	multiplicity of infection
O.D.	optical density

P.B.S.	phosphate buffered saline (Dulbecco and Vogt, 1954)
P.B.S.(CMF)	calcium-magnesium-free P.B.S.
P.F.U.	plaque-forming unit
p.i.	post-infection
RI	replicative intermediate
RNA	ribonucleic acid
rpm	revolutions per minute
2^{o}	secondary
S	svedberg sedimentation unit
SDS	sodium dodecyl sulphate
SSC	standard saline citrate (Marmur, 1961)
SV_{40}	simian virus 40
TC	tissue culture
TCA	trichloroacetic acid
TdR	thymidine
TSP1	Toronto small plaque variant of polyoma virus (Stanners, 1963)
UV	ultra-violet
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

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INTRODUCTION

I. THE POLYOMA VIRION

Polyoma virus was first discovered by Gross, who injected C_3H mice with cell-free extracts from the tissue of AKR mice which had spontaneous leukemia (Gross, 1953). He observed that some mice developed leukemia while others developed tumors on one or both sides of their neck (Gross, 1953; Stewart, 1953).

The virus has been variously referred to as: "filtrable agent" (Gross, 1953), "parotid tumor agent" (Lindqvist & Sinsheimer, 1967; Joyner *et al.*, 1966), "substance" or "tumor-inducing agent" (Stewart *et al.*, 1957), "SE polyoma virus" (Eddy, Stewart & Grubbs, 1958), "mouse tumor virus (SE polyoma)" (Rowe *et al.*, 1958), "P virus" (Buffet *et al.*, 1958), "parotid tumor agent (polyoma virus)" (Banfield, Dawe & Brindley, 1959), "Mill Hill polyoma virus" (Negroni, 1960), and "polyoma T or Toronto virus" (Sheinin, 1962). Virus preparations from tissue culture passages were capable of inducing a wide variety of solid neoplasms in mice, rats, hamsters, rabbits, guinea pigs, mastomys, and ferrets, hence the name polyoma (Stewart & Eddy, 1959).

Tumor induction may be achieved via many innoculation routes including subcutaneous, intramuscular, intra-peritoneal, intravenous, intraspinal, intranasal, oral, intracerebral, intra-cerebellar, heart, salivary glands, and *in utero* (Eddy, 1969). Polyoma and simian virus 40(SV40) are minute animal viruses with a sedimentation coefficient of 240 $S_{20,w}$ (Kahler, *et al.*, 1959). Polyoma was the first DNA animal virus to be crystallized, and the infectious particles have a buoyant density of 1.34 g/ml while the empty capsids have a buoyant density of 1.29 g/ml in cesium or rubidium chloride (Kahler *et al.*, 1959) and an icosahedral capsid with 72 capsomeres. In negatively stained preparations, it appears roughly spherical with a diameter of 45 mu. (Howatson and Almeida, 1960; Wildy et al.,1960; Crawford, Crawford and Watson, 1962.)

There are many isolates in existence and none of these possess any major detectable genetic variations. They fall into two different plaque morphological groupings: a large or small plaque variant. For the present work, only the small plaque isolate of Toronto strain TSP1 was used (Stanners, Till & Siminovitch, 1963).

Four different types of particles can be isolated from polyoma infected cultures. (i) The most commonly encountered are infectious full particles with a full complement of the polyoma form I DNA. (ii) Defective particles, *i.e.* the encapsidated polyoma genome has in its DNA either a deletion, rearrangement, substitution,

duplication, or insertion. In most cases, the genome is less than unit length. The deletions are of different sizes (Thorne, 1968; Blackstein, Stanners & Farmilo, 1969). Defective polyoma DNA covalently linked to host sequences has been found in serially passed viral stocks (Lavi & Winocour, 1974). Several recent works have shown that defective variants of Polyoma (Folk & Wang, 1974) and SV_{40} (Brockman, Lee & Nathans, 1974; Winocour *et al.*, 1974) have reiterations of viral initiation sites, either in duplicate or in multiple copies. (iii) Pseudovirions are particles containing form FII DNA, that is, linear random fragments of host DNA sedimenting at 14S or less under neutral conditions (Michel, Hirt & Weil, 1967; Winocour, 1968, Osterman, Waddell & Aposhian, 1970; Qasba & Aposhian, 1971; Yelton & Aposhian, 1972). Pseudovirions are invariably found in virus infected cultures and amount to about 20-40% of the particles depending upon the cell type used and multiplicity of infection (Crawford, 1969; Winocour, 1969). Data obtained from studies on SV40 seem to indicate that induction of the synthesis and fragmentation of cellular DNA is required for the formation of pseudovirions (Ritzi & Levine, 1970). (iv) The fourth type of particle encountered and the second most common are empty capsids. These have no infectivity as a result of the absence of nucleic acid.

II. CLASSIFICATION

Polyoma virus belongs to the family Papovaviridae, which contains two genera: Papillomavirus and Polyomavirus. The genera differ primarily in genome size. The cryptogram for the Polyoma genus was (D/2:3/7-13 s/s:v/o) (Wildy, 1971). Recently, the study group on Papovaviridae created by the Vertebrate Subcommittee of ICNV was requested by the Executive Committee to reconsider, in particular, the name of the genera. The suitability of Polyomavirus as the generic name was to be re-evaluated because of it's similarity to the name of the murine member of the group. The subcommittee proposed that the two Papovaviridae genera be designated Papovavirus A and Papovavirus B in conformity with the rules of ICNV (Melnick *et al.*, 1974). The same study group proposed that polyoma virus be designated as type 1, Simian Vacuolating virus (SV_{40}) designated as type 2, K-Papovavirus as type 3, rabbit kidney vacuolating virus (RKV) as type 4, BK virus from human urine following renal transplantation as type 5, and JC virus from progressive multifocal leucoencephalopathy patients as type 6. The genus cryptogram now stands as (D/2:2 4-3/12:s/s:v/o).

III. THE PHYSIOLOGY OF VIRUS INFECTION

Virus-cell interactions ellicited by polyoma virus are of two general types. The common occurrence is

a productive infection in which the virus replicates inside the cell, killing the cell in the process; this is also called the lytic cycle. Dependent upon the cell type, the infection ellicited could be incomplete, abortive, or non-productive where the virus fails to complete the replication cycle. When this abortive infection generates a stable inheritable trait, it is called transformation. However, if the growth characteristics change only temporarily and returning to normal after several generations this is termed abortive transformation. The response of mouse cells is primarily that of productive infection although low frequency tranformation has also been detected. Transformation is usually associated with rat and hamster cell cultures.

IV. EARLY STAGES OF POLYOMA INFECTION

A number of laboratories have attempted to follow the lytic events of polyoma virus infections in various culture systems (Bourgaux, 1964; Mattern, Takemoto and Daniel, 1966; Maurer, Wertman and Yall, 1969.) Electron microscopic observations have indicated that the adsorption and penetration steps are similar to other viruses (i.e. via viroplexis). Recently, Frost completed a detailed study on the decapsidation sequence of events for both wild type and a number of early and late temperature-sensitive mutants of polyoma (Frost, 1975.) He overcame most of

the previously existing problem by increasing the sensitivity of the assay system by using ^{125}I and ^{131}I, which yielded a 100-fold higher specific activity than that afforded by ^{3}H-labeled amino acid in the capsid. He proposed that histones on the external surface of the virus particle began to dissociate away from the capsid outside the cell prior to the actual penetration of the virion through the cell membrane. Once within the cytoplasm, the virus particle started its decapsidation by a further loss of VP4-6 histone-like protein moieties, generating sub-viral decapsidation intermediates (Frost, 1975). The released naked viral DNA was later transported across the nuclear membrane (Khare & Consigli, 1965; Frost, 1975). It is well known in the bacteriophage system that the DNA of temperate phages can exist in two states: the vegetative phage and the prophage. Also, the F factor and other bacterial episomic factors such as the resistance-transfer factors are all capable of either autonomous replication, remain in the integrated state, or shift between these two alternative pathways. Polyoma (Babiuk & Hudson, 1972; Ralph & Colter, 1972; Lavi & Winocour, 1974) and SV_{40} (Rhoades & Thomas, 1968; Tai *et al.*, 1972; Holzel & Sokol, 1974) DNA do, in fact, integrate into the host genome after the viral DNA enters the cell nucleus. This integration step may be obligatory in productive infections (Weil & Kara, 1970; Ralph & Colter, 1972).

V. VIRAL DNA

1. General Considerations

DNA isolated from virions can be grouped into form I, form II and form III (see Fig. 1 & Fig. 2). The three forms of DNA sediment under neutral pH with sedimentation coefficients of 20S, 16S, and 14S (Weil, 1963; Weil *et al.*, 1967), also see Materials and Methods Fig. 1 & Fig. 2). Form I DNA is double stranded and covalently closed (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963; Dulbecco, 1964; Crawford *et al.*, 1964; Vinograd *et al.*, 1965). Both form I and II have a buoyant density of 1.709 g/ml in CsCl while form III DNA is only 1.702 g/ml due to its cellular origin (Michel, Hirt & Weil, 1967). After a single break was introduced into the form I molecule, this DNA was observed to sediment at only 16S instead of its usual 20S (Weil & Vinograd, 1963). Purified Polyoma DNA retains its infectivity as well as its tumor inducing activity (DiMayorca *et al.*, 1959) and the amount of infectious DNA in the virion amounts to 10-12% of the total content by weight (Winocour, 1963; Molteni *et al.*, 1966).

The molecular weight of Polyoma DNA is estimated to be 3×10^6 daltons (Crawford, 1964; Weil *et al.*, 1967; Gray, Upholt, & Vinograd, 1971) with a G + C content of approximately 48% (Crawford, 1962; Crawford *et al.*, 1964) to 49% (Gray, Upholt & Vinograd,

Fig. 1. Schematic representation of various forms of DNA isolated from polyoma virus infected cell cultures.

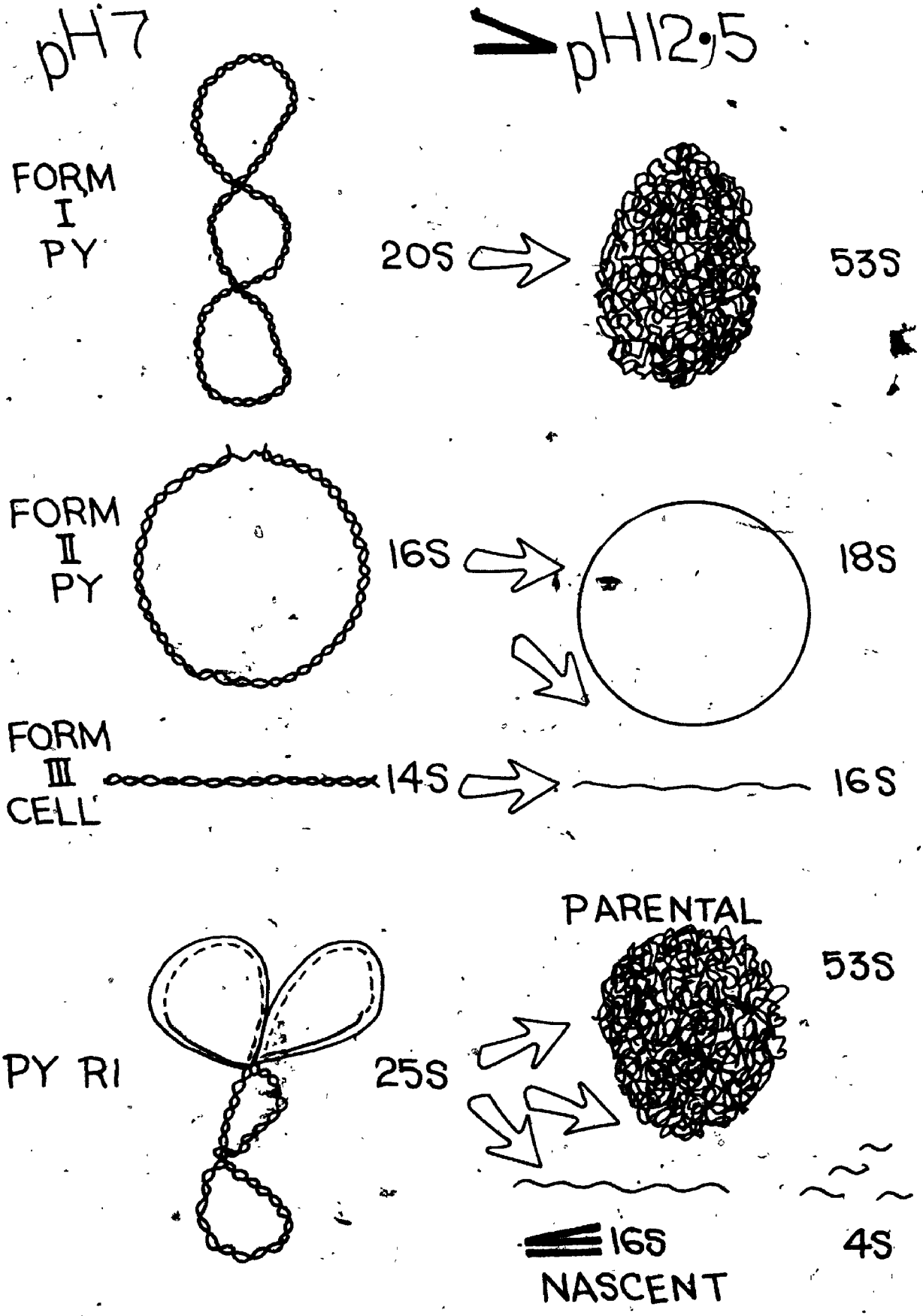

pH7
pH12,5
FORM I PY
20S
53S
FORM II PY
16S
18S
FORM III CELL
14S
16S
PY RI
25S
PARENTAL
53S
16S NASCENT
4S

Fig. 2. Representative distribution of various nucleic acids neutral or alkaline sucrose velocity sedimentation analysis.

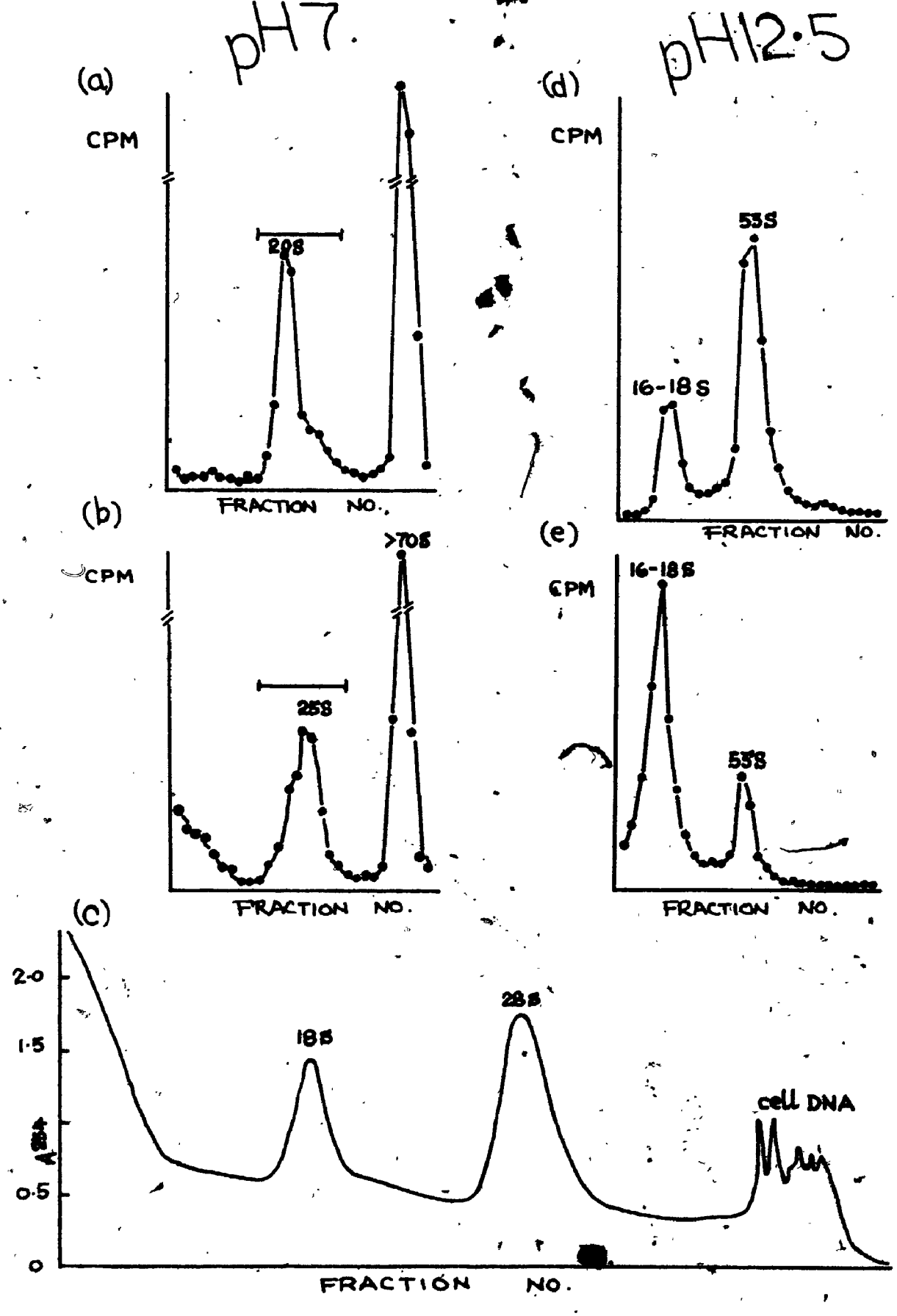
a) Radioactivity distribution after long labeling of polyoma infected cells with [^{3}H] TdR analysed under neutral conditions.

b) Radioactivity distribution after short pulse labeling of polyoma infected cells with [^{3}H] TdR analysed under neutral conditions.

c) A^{254} tracing of ribosomal RNA 18S & 28S of polyoma infected cell cultures analysed under neutral conditions.

d) material in a) analysed under alkaline conditions.

e) Short pulse DNA isolated from the indicated RI region in b) reanalysed under alkaline conditions.


pH7
(a)
CPM
20S
FRACTION NO.
(b)
CPM
>70S
25S
FRACTION NO.
(c)
2·0
1·5
0·5
0
A 254
18S
28S
cell DNA
FRACTION NO.
pH12·5
(d)
CPM
53S
16-18S
FRACTION NO.
(e)
CPM
16-18S
53S
FRACTION NO.

1971). The relative amount of the four nucleotides is as follows: dCMP,23: dAMP,23:dGMP,19: dTMP,25 (Smith *et al.*, 1960). DNA of this size (assuming SV40 and Polyoma are the same) has approximately 6000 base pairs with a coding potential sufficient for 2000 amino acids or 10 proteins of 20,000 daltons. Its unit length as measured by electron microscopy with rotary shadowing ranges from 1.41-2.0 μ (Tooze, 1973).

Polyoma DNA was the first DNA reported to occur as a natural circular duplex molecule (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963). So far, all such molecules found have a superhelical tertiary structure. As a consequence of the special tertiary structure, there are a number of unusual features which give this superhelical circular duplex DNA its characteristic properties (Clayton & Vinograd, 1967; Crawford & Waring, 1967; Hudson & Vinograd, 1967; Radloff, Bauer & Vinograd, 1967; Wang, Baumgarten & Olivera, 1967). Among these are: a decreased intrinsic viscosity (Opschoor, 1968), decreased sensitivity to shear (Kleinschmidt, Burton & Sinsheimer, 1963; Weil & Vinograd, 1963; Ogawa & Tomizawa, 1967; Young & Sinsheimer, 1967; Rhoades & Thomas, 1968) and the unique capability of spontaneous renaturation after heating of the DNA to 100°C (Weil, 1963). Also, after alkaline denaturation dependent upon the integrity of the supercoil, it may sediment as a single-stranded linear

piece of DNA at 16S or as 18S single-stranded circular DNA if a single-strand break was introduced. The intact supercoil, after exposure to alkali, will sediment rapidly at 38-53S as a collapsed DNA mass (Vinograd *et al.*, 1965; Vinograd & Lebowitz, 1966; Vinograd, Lebowitz & Watson, 1968; Fig. 2(d) & (e)). Beside the fact that polyoma DNA strands do not separate with ease due to its covalent circularity, it has also demonstrated a decrease in its ability to take up intercalating dye molecules such as ethidium bromide or its analogue propidium diiodide. Intercalation causes partial unwinding of the duplex structure. For form I DNA, a critically small amount of dye can reduce the number of left-handed superhelical turns to zero (i.e. it will co-sediment with form II DNA) while further dye-binding will result in the formation of superhelices in the opposite sense (Crawford & Waring, 1967; Radloff, Bauer & Vinograd, 1967; Wang Baumgarten & Olivera, 1967; Bujard, 1968; Bauer & Vinograd, 1968; Ruttenberg *et al.*, 1968). Thus the superhelical DNA rapidly becomes wound into a tight conformation which can no longer bind intercalating dye molecules. Since polyoma DNA contains less than the regular Watson-Crick type number of turns per base pair, when the last internucleotide bond is completed, the molecules assume a right-handed superhelical configuration (Vinograd *et al.*, 1965). The number of superhelical turns in the molecule has been estimated to

be 15-20 from alkaline denaturation studies (Vinograd & Lebowitz, 1966) and about 12 from dye-binding estimations (Crawford & Waring, 1967). The relationship between superhelical turns and the Watson and Crick helical turns is given by the equation:

$$T = \alpha - \beta$$

where α is the topographical number or the revolutions made by one strand about the duplex axis when the axis is constrained to a plane. β is the duplex winding number or the revolutions made by one strand about the duplex axis when it is not constrained to a plane. T is the superhelix winding number or the number of revolutions made by the duplex about the superhelix axis (Vinograd & Lebowitz, 1966; Vinograd, Lebowitz & Watson, 1968). Superhelix density (σ) is referred to the number of superhelical turns per 10 base pairs (Vinograd, Lebowitz & Watson, 1968):

$$\sigma \equiv \frac{T}{\beta^{o}}$$

β^{o} is numerically equal to 1/10 base pairs per molecule. The superhelix density of polyoma DNA was reported to be $-3.3 \pm 0.3 \times 10^{-2}$ (Gray, Upholt & Vinograd, 1971) in 2.83 M CsCl.

Although the genome size of polyoma DNA is

small, progress of its map was rather unfruitful until the recent employment of various sequence specific bacterial restriction endonucleases. Finally, the ordering of various restriction enzyme cleavage fragments has led to the formation of the physical map of polyoma DNA (Griffin, Fried & Cowie, 1974, Fig. 3). Another polyoma physical map is also available. This second map was constructed by ordering of fragments produced by yet another restriction enzyme from *Haemophilus aegyptius*, Endonuclease R. HaeIII (Summers, 1975).

2. Replication of polyoma DNA

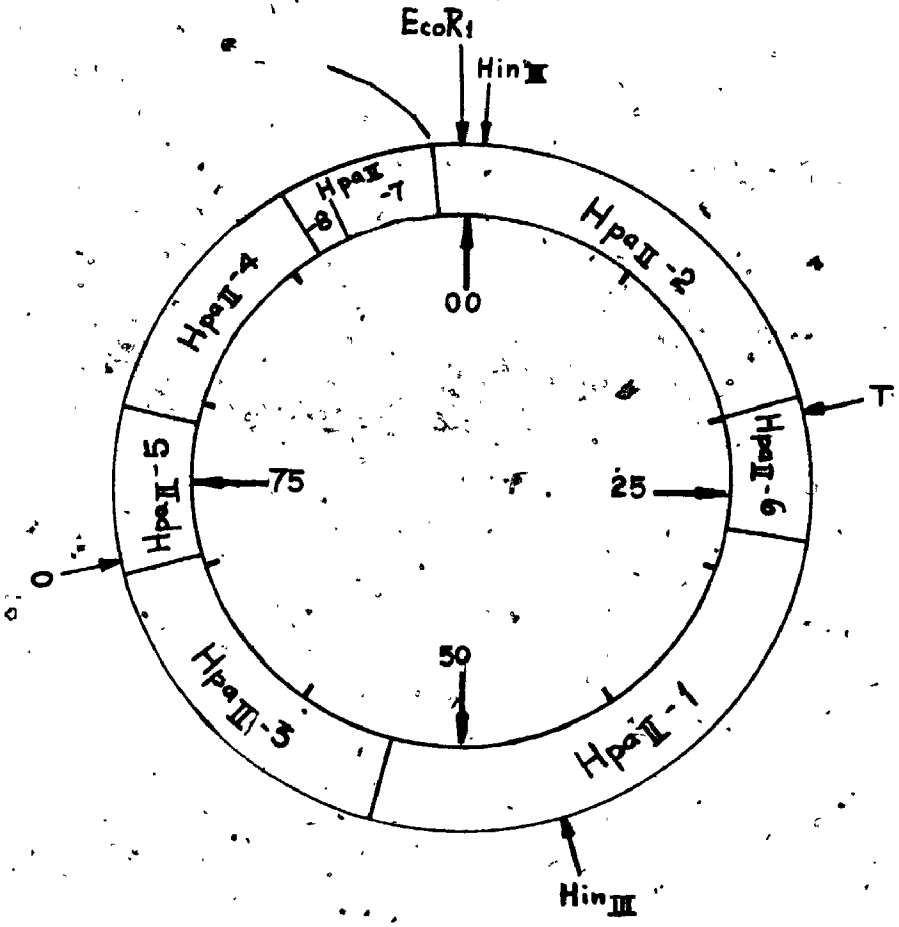
Polyoma virus DNA replicates in the nuclei of permissive cells (Minowada & Moore, 1963) and was found *in vivo* (Hirt, 1966) in isolated nuclei (Winnacker, Magnusson & Reichard, 1971,1972; Magnusson *et al.*, 1972; Pigiet *et al.*, 1973) and *in vitro* (Hunter & Francke, 1974b) to follow a semiconservative mode of replication. Further studies have led to the identification of polyoma replicative intermediate molecules (Bourgaux, Bourgaux-Ramoisy & Dulbecco, 1969; Bourgaux, Bourgaux-Ramoisy & Seiler, 1971). Using electromicroscopic techniques, triple-branched replicating molecules with dual branch points and no ends were seen (Hirt, 1969) as predicted by the Cairn's model for closed circular DNA replication

(Cairns, 1963). Soon after the interesting discovery that Simian virus 40 DNA replicates without any discontinuities in the parental strands (Jaenisch, Mayer & Levine, 1971; Sebring *et al.*, 1971), similar configurations were also visualized for polyoma replicative intermediates. These molecules were conspicuously characterized by their retainment of superhelical structures at the still unreplicated regions of the parental DNA (Widner, 1971; Meinke & Goldstein, 1971; Bourgaux & Bourgaux-Ramoisy, 1972b, Roman Champoux & Dulbecco, 1974). It is because the parental DNA strands in the replicative intermediates are covalently closed that the idea of a swivel at the unreplicated region of the DNA seem particularly attractive. One of the simplest forms of a swivel can be repeated cycles of nicking (as by an endonucleolytic activity), unwinding, and followed by rapid reclosing, such as the action of a polynucleotide ligase, (Sebring *et al.*, 1971; Bourgaux & Bourgaux-Ramoisy, 1972a, Roman, Champoux & Dulbecco, 1974), or by the action of the DNA untwisting enzyme (Champoux & Dulbecco, 1972). As a result of such an unusual mode of replication, RI molecules exhibit an extraordinarily rapid rate of sedimentation in a neutral sucrose velocity analysis. RI molecules are commonly reported to sediment heterogeneously at neutral pH with a higher sedimentation coefficient than form I DNA. The peak of distribution centres at about 25S (Meinke & Goldstein, 1971; Bourgaux, Bourgaux-Ramsoisy & Seiler,

1971; Magnusson, 1973; Francke & Eckhart, 1973; Roman, Champoux & Dulbecco, 1974; see also Fig. 2(b)). This was substantiated by *in vitro* studies (Hunter & Francke, 1974a) and experiments using isolated nuclei (Winnacker, Magnusson, & Reichard, 1971,1972; Magnusson *et al.*, 1973; Pigiet *et al.*, 1973). From single stranded specific nucleolytic digest studies, the replication of polyoma DNA was found to follow a bidirectional synthetic scheme (Bourgaux & Bourgaux-Ramoisy, 1971). This was later confirmed by two different reports following the popular utilization of restriction enzymes as cleavage markers. Modelling after Fareed and coworkers (Fareed, Garon & Salzman, 1972), Crawford used *E. coli* R_1 specific endonuclease and established that indeed both large and small plaque polyoma viral DNA, like SV40, also replicate bidirectionally. Initiation of DNA synthesis appears to be at a fairly specific site. More current was the work of Griffin *et al.* (Griffin, Fried & Cowie, 1974, also see Fig. 3). These workers have located the precise origin and terminus for polyoma DNA replication using three different restriction enzymes' cleavage sites as markers. They localized the origin of DNA replication at 71± 3 map units from the $EcoR_1$ cleavage site: probably in HpaII-5 and very close to the HpaII-2-HpaII-6 junction since replication was bidirectional and synthesis in both directions takes place at about equal rates. Beginning with Okazaki's observation on a discontinuous mechanism of DNA synthesis

Fig. 3. The physical map of polyoma DNA (A-2 strain) as determined by Griffin *et al.* (1974) using restriction enzymes.

The order of fragments are as shown. The EcoR1 cleaves at 00 map units and the origin (O) at 79 and termination (T) of DNA replication is at 21 map units.


EcoR1
Hin III
Hpa II-2
Hpa II-6
Hpa II-1
Hin III
Hpa II-3
Hpa II-5
Hpa II-4
Hpa II-7
Hpa II-8
00
25
50
75
T
0

for *E. coli* (Okazaki *et al.*, 1968a,b; Sugino, Hirose & Okazaki, 1972) short fragments of 100 to 140 deoxyribonucleotides long were also found *in vivo* (Magnusson, 1973) polyoma DNA replication as well as in isolated nuclei (Magnusson *et al.*, 1973; Pigiet *et al.*, 1973). As in the microbial systems (Brutlag, Schekman & Kornberg, 1971; Schekman, *et al.*, 1972; Sugino, Hirose & Okazaki, 1972), these short "Okazaki fragments" were found initially as covalently-linked RNA-DNA polynucleotides (Magnusson *et al.*, 1973). The priming of DNA synthesis by short stretches of RNA were soon confirmed by *in vivo* studies. The RNA was found to be linked to the 25S RI molecules (Sadoff & Cheevers, 1973). In various isolated nuclei and *in vitro* studies, RNA was linked to the 5' end of the 4-5S pieces with a 3'⟶5'-diester linkage (Sadoff & Cheevers, 1973; Magnusson *et al.*, 1973; Pigiet *et al.*, 1973; Francke & Hunter, 1974a). This RNA primer, in contrast to the more stable and much longer RNA primer of the *E. coli* system (Sugino, Hirose & Okazaki, 1972) is at most 30 bases long (Hunter & Francke, 1974a). Its link to the deoxyribonucleotides is apparently of a transient nature (Pigiet *et al.*, 1973; Hunter & Francke, 1974a) as the RNA is subsequently removed, presumably by an RNAse-H activity (Hausen & Stein, 1970), before the neighbouring DNA strand was elongated by a gap-filling DNA polymerase. The final ligation was by a DNA ligase forming the last phosphodiester bond, as the DNA chain grows. The transition from

RNA to DNA appears to be base-specific in some systems (Hirose, Okazaki & Tamanoi, 1973; Sugino & Okazaki, 1973) such as the unique sequence p(rPy)-p(dC)p for *E. coli* (Hirose, Okazaki & Tamanoi, 1973) but not in ϕX174 (Wickner *et al.*, 1972) or polyoma (Magnusson *et al.*, 1973; Pigiet *et al.*, 1973; Hunter & Francke, 1974 a). Although there is much information on RNA primed short "Okazaki fragment" synthesis in the literature, two areas remain unresolved for polyoma: one group, working with isolated nuclei, found evidence supporting a mechanism of total discontinuous replication, i.e. discontinuous replication for both strands of polyoma DNA (Pigiet *et al.*, 1973) while another group reports on a semidiscontinuous mechanism of replication (Francke & Hunter, 1974a) i.e. discontinuous replication for only one of the two strands. The major discrepancy between the findings of the two groups is the degree of self-complementarity of the short pieces of newly synthesized DNA. The proponents for total discontinuity observed 4S strands self-annealed with high efficiency up to 80%, strongly supporting discontinuous replication for both daughter strands at the replication forks (Pigiet *et al.*, 1973). On the other hand, short chains newly synthesized *in vitro* showed only 20% self-complementarity (Francke & Hunter, 1974a). Moreover these workers observed differences in labelling ratio between short chains and long chains (up to unit genome length) unless radioactivity was administered for a shorter

duration than the life time of a single "Okazaki fragment". Under this latter labelling scheme, about equal amounts of radioactivity are incorporated into long chains as into short fragments. This is in strong support of a semi-discontinuous mode of DNA replication (Hunter & Francke, 1975). The second unresolved area pertains to polymerases in polyoma DNA replication. This is a general rather than a special area which needs to be further investigated. There exist many similarities between the mechanisms of *E. coli* DNA chain growth and that of polyoma DNA. It also does not seem unlikely that polyoma DNA replication utilized cellular enzymes and factors for chain growth. In fact, there may be an universal replication mechanism for both procaryotes and eucaryotes. In *E. coli*, there are at least three DNA polymerases known (Sutton, 1971) and a similar number for mammalian cultured cells was reported (Chang & Bollum, 1972). With respect to polyoma, Magnusson and coworkers detected at least two DNA polymerase activities by their display of differential sensitivity to the inhibition of hydroxyurea (Magnusson *et al.*, 1973). More recently, from the sensitivity pattern of 1-β-D-arabinofuranosyl CTP, at least three different DNA polymerase activities were segregated in an *in vitro* polyoma DNA synthesizing system (Hunter & Francke, 1975).

VI. INTERNAL ENZYMES

Cuzin and co-workers began their search some years ago for a site specific enzyme relating to processes such as initiation and termination of viral DNA replication, and/or integration into and excision from the host cell DNA (Stoker & Dulbecco, 1969; Cuzin et al., 1970). They subsequently reported a specific endonuclease associating with the virus (Cuzin, Blangy & Rouget, 1971; Cuzin, Rouget & Blangy, 1973). After considerable care, these workers confirmed the existence of an internal enzyme free from suspicions of merely being a trivial contaminant (Parodi et al., 1974). This enzyme is a site specific endonuclease. It cleaves at one unique A-T-rich region in the polyoma form I DNA less than 0.05 genome length from the Eco R_1 site. Although the activity of this enzyme was reported to be missing from both Ts-A and ts-25 of the same early complementation group (Parodi et al., 1974), it is still not fully established whether this enzyme is a viral or cellular gene product.

VII. BACKGROUND AND OUTLINE OF PRESENT RESEARCH

Cell division occurs after genome replication. In the case of normal cells, the division in vitro is strictly controlled and the cells spread over the culture substrate until they reach a critical "saturation density", at which time division slows down and stops. Tumour cells however do

not generally exhibit clear cut cell density dependent growth inhibition and they continue to divide until very high saturation densities are attained. Uncontrolled proliferation and spread is characteristic of cancer: a sizable obstructive tumour may originate from a single microscopic cell eventually proliferate into millions of neoplastic cells each of which is capable of separating from the original mass, migrating and re-establishing a colony at a new location. The mechanism by which this is achieved is yet not understood. Uncontrolled cell division could be the result of either of the following mechanisms: the lack of controlling factor(s) which operates in the normal cells to limit proliferation, failure of a cell to respond to a message to stop generation, or having acquired a new mechanism not present in normal cells enabling it to continuously proliferate. The control of cell division is possibly exerted by regulating the replication of DNA. In view of this, the study of the regulation of DNA replication may result in the following: (i) the basic understanding of the process of replication of the fundamental genetic material, (ii) elucidation of the various events which control genome expression and, (iii) identification of the viral gene function(s) required for neoplastic transformation of cells.

There is growing evidence that many types of cancer may have a viral etiology. As a result, many

workers have used oncogenic viruses as model systems because of their distinct advantages over all others. Among these, two members of the papovavirus group, SV_{40} and polyoma virus, are especially well studied. Reasons for their selection as study models are as follows: (i) they can be easily cultured and assayed accurately in tissue culture, (ii) their relatively small genome size of 3×10^6 daltons codes for a limited number of polypeptides, (iii) they have the ability to induce neoplastic transformation and, (iv) they interact with the host cell altering its control and function.

Regulation of genome replication has been well studied in several bacterial phage systems such as ϕX174 (Levine & Sinsheimer, 1968, 1969a), λ (Levine & Sinsheimer, 1969b), and S13 (Tessman, 1966) while no such comparable study has been done with mammalian viruses. Much evidence points toward the existence of regulatory factor(s) which was defined and studied through the use of temperature sensitive mutants (Eckhart, 1974). At least in procaryotic interactions, viral determinants are involved as well as host coded regulatory factors (Littlefield & Basilico, 1966; Basilico, Matsuya & Green, 1969) Particular cistrons responsible for DNA replication have been localized e.g. cistron VI in ϕX174 (Linqvist & Sinsheimer, 1967; Sinsheimer, 1968; Levine & Sinsheimer, 1968), cistron IV for S13 (Tessman, 1966), the more well known O and P genes in the case of λ (Brooks, 1965; Joyner et al., 1966; Dove, 1968; Pereira

et al., 1968), and the N cistron responsible for the indirect regulation (Brooks, 1965; Eisen *et al.*, 1966; Joyner *et al.*, 1966; Packman & Sly, 1968; Sly, Eisen & Siminovitch, 1968; Eisen *et al.*, 1968). As many as 20 or more genes are known to be responsible for the genesis of T_4 DNA. Some of these have already been isolated and characterized (Wiberg *et al.*, 1962; Dewarrd, Paul & Lehman, 1965; Warner & Barnes, 1966a,b; Greenberg, 1966; Wilberg, 1966; Duckworth & Bessman, 1967; Fareed & Richardson, 1967; Weiss & Richardson, 1967). Most of these are not DNA polymerase or DNA precursor products.

Ts mutants affecting polyoma genome replication have been well studied and showed that neither in the case of bacteriophage nor with polyoma, was polymerase activity or DNA precursor production involved (Kohiyama *et al.*, 1963; Bonhoeffer, 1966; Pauling, 1968; Fangman & Novick, 1968; Hirota, Ryter & Jacob, 1968).

The present research takes its cue from the findings that both adenovirus (Polasa & Green, 1965) and a close relative of polyoma, SV_{40} (Kit *et al.*, 1969), require concurrent protein synthesis for their viral DNA replication although the exact functions of these proteins are unknown. Later, Branton, Cheevers & Sheinin (1970) found that the step in polyoma DNA maturation is blocked along with aberrent cell DNA synthesis late in the

infection cycle when protein synthesis is inhibited by treatment with cycloheximide. A new DNA sedimenting heterogeneously at 16-20S was also recovered. Another group, using SV40, established that different proteins are required for the initiation and closure steps of SV40 DNA replication (Kang *et al.*, 1971). It was the intention of the author to verify various claims in these reports using polyoma infected 2° mouse embryo cell cultures and to further extend their findings by elucidating the various controlling mechanisms in polyoma DNA replication either in the presence or absence of *de novo* protein synthesis. Special attention was paid to correlate particular stages of viral DNA replication and their individual requirements for *de novo* protein synthesis. In Part I of the Results section, experiments are described which were designed to elucidate the exact nature of DNA synthesis inhibition induced by cycloheximide block. Following this set of experimental designs, work subsequently described concentrated on the tertiary structure of the residual DNA synthesized under the influence of the drug. After the author's attempt to define the physico-chemical nature of the newly-synthesized residual DNA, various experiments on the biochemistry of polyoma DNA replication followed. Both the forward generation of DNA under the influence of cycloheximide as well as the recovery of DNA synthesis after antibiotic removal was described and, in

particular, decay and regeneration kinetics for the various regulatory factors were detailed. The findings are summarized in a schematic diagram included in the Discussion where further experimentation is also suggested.

MATERIALS AND METHODS

(1) Cell Cultures

i) Cultivation of primary mouse embryo cell culture

Inbred white swiss mice from Carworth Farms, New City, New Jersey, U.S.A. were sacrificed by spinal dislocation on their 14-17th day of pregnancy. After the embryos were taken out of the uterus using aseptic technique, they were decapitated, minced; limbs and tails were dismembered. Cells were disaggregated by a trypsin-EDTA solution. Monodispersed cells were seeded into 75 cm^2 FALCON 3024 bottles at a density of 7.5×10^6 viable cells per bottle. The seeding medium is modified McCoy 5A medium supplemented with fetal calf serum (FCS) (10% v/v), penicillin (100 units/ml), streptomycin (100 ug/ml) and Fungizone (5 ug/ml); all expressed as final concentrations. Incubation was at 37°C with a 95% air-5% CO_2 gas phase. Under these conditions, the primary cultures generally become confluent in approximately 5 days after seeding. Subculturing into secondary cultures was usually performed when the 175 cm^2 bottles had reached a total viable population of 1.0×10^7. This corresponds

to 7 days post seeding, approximately.

ii) Preparation of secondary mouse embryo cell culture for experiments

Primary cultures were trypsinized with a trypsin-EDTA solutions when the monolayers were ready for subculture. Disaggregated cells were resuspended in modified McCoy 5A medium supplemented with FCS (10% v/v), penicillin (100 units/ml), and streptomycin (100 ug/ml); all expressed as final concentrations. Each 60 cm^2 FALCON 3002 petri plate was seeded with 1.2 x 10^6 cells. At this seeding density, plates usually require 3-4 days to reach confluency. They were allowed to divide further and were taken for experiments when they reached 7.5-8 x 10^6 cells per plate (approximately 4-5 days post subculture).

(II) Preparation of high titre polyoma virus stock

In order to obtain a high titre (10^8-10^{10} PFU/ml) virus stock for experimentation, the procedure reported by Winocour was employed (1963) Crude lysates were used without further purification and storage of the stock was at -10°C.

(III) Virus Infection of cell cultures

Infection of secondary cell cultures by polyoma

virus TSP-1 was achieved by aspirating the medium from the culture plates before a small volume of a concentrated virus stock was placed to infect the cells at a multiplicity of infection (m.o.i.) at about 50. Similar protocol was employed in the case of mock-infected cultures for control purposes except that an equal volume of medium was administered in place of the stock virus.

Polyoma virus was allowed to adsorb to the cell surface in a 95% air-5% CO_2 gas phase at 37°C for 90 minutes before fresh modified McCoy 5-A medium supplemented by FCS (1% v/v) and antibiotics was replaced. Individual experimental manipulations are described separately later.

(IV) Labeling or pulse-chase of viral DNA

Cultures actively replicating viral DNA were usually pre-exposed for 60 min. to medium with no supplement of FCS before continued exposure to such a medium containing methyl ^{3}H-thymidine ([^{3}H]TdR). The conditions of labeling are given in the individual experiments. For pulse-chase experiments, the labeled culture medium was decanted; the monolayers were rapidly washed twice with prewarmed (37°C or 23°C; dependent upon the temperature of the chase) medium containing 2×10^{-5}M

of unlabeled thymidine (TdR) and then further incubated with a fresh change of the same medium for the desired chase duration.

(V) Drug treatment and subsequent reversal of its action.

In all experiments CX was prepared fresh just before use in modified McCoy 5A medium with the antibiotic supplement but in the absence of any FCS. A CX stock of 10 mg/ml was routinely employed. In all experiments the stock solution was diluted to 1 mg/ml before the correct amount was administered to the cultures to make a final concentration of 10 ug/ml. For the purpose of reversing the action of CX, cultures were washed thoroughly with plain modified McCoy 5A medium supplemented only with antibiotics, but devoid of either FCS or CX. If a chase of the incorporated radioactivity is desired during the course of CX reversal, modified McCoy 5A medium was further supplemented with 20 uM of unlabeled TdR and cells were exposed to this medium for the desired duration.

(VI) Isolation of viral DNA from virus infected cells

After each period of labeling or chase, monolayers were washed rapidly once with ice-cold standard saline citrate (Marmur, 1961) (SSC) and monodispersed by treatments with 0.1% trypsin in saline-citrate solution

(Sheinin, 1961). Cells were then washed again with SSC by low speed centrifugation and lysed by addition of 0.1 volume of 10% sodium dodecyl sulfate (SDS). The SDS-lysates were sedimented without further purification through 15-30% (w/w) sucrose gradients in neutral EDTA buffer containing 0.5% SDS (Penman, Vesco & Penman, 1968).

(VII) Analyses of radioactively labeled DNA

1. Velocity sedimentation in neutral sucrose density gradients of radioactively-labeled DNA from whole cell lysates.

SDS-lysates were sedimented through a 30-ml 15-30% (w/w) sucrose gradient in neutral EDTA buffer containing 0.5% SDS, formed over a 6-ml 70% (w/v) sucrose cushion also in EDTA buffer pH 7.4. Centrifugation was at 26,000 rpm at 23°C for 10 hours using a Spinco SW27 rotor. Under these conditions approximately 95% of the cellular DNA in the crude SDS-lysates is recovered as high-molecular-weight material (>70S), which sediments through the gradient and is collected on a 70% sucrose cushion (Branton, Cheevers & Sheinin, 1970; Branton & Sheinin, 1970). Polyoma DNA components are resolved within the gradient (Cheevers, 1973; Cheevers, Kowalski & Yu, 1972; Yu, Kowalski & Cheevers, 1975).

Viral DNA was recovered for subsequent analysis

as follows. The appropriate neutral gradient fractions were combined and mixed with 2.5 volumes of cold 95% ethanol. After overnight storage at -15°C, the DNA was sedimented (10,000 x g, 30 minutes, 0°C) and dissolved in Buffer A (Kiger & Sinsheimer, 1969).

2. Velocity sedimentation in alkaline sucrose density gradients of isolated radioactively labeled DNA.

Form I polyoma DNA was separated from alkali-denaturable RI by velocity sedimentation at alkaline pH. Viral DNA was denatured by addition of 0.1 volume of 1.0 N NaOH-0.01 M EDTA and centrifuged in 15-30% (w/w) sucrose gradients in SA buffer with 0.1% lauroyl sarcosine (SW 27 rotor, 26,500 rpm, 8 hours, 23°C).

The size distribution of nascent viral DNA strands derived from BND-cellulose purified RI (see VII, 4) was determined by sedimentation through 10-30% (w/w) alkaline sucrose gradients in LA buffer with 0.1% N-lauroyl sarcosine (SW 40 rotor, 39,500 rpm, 18 hours, 23°C). Molecular weight distributions were determined according to the formula, $\text{Log}_{10}M=(\text{Log}_{10}S + 1.277)/0.4$, where M = molecular weight in daltons and S = sedimentation coefficient in 1 M Na^+ (Studier, 1965). The sedimentation marker was 18s ribosomal RNA centrifuged in parallel 10-30% (w/w) sucrose gradients in neutral LA buffer with

0.1% lauroyl sarcosine.

3. Isopycnic dye-buoyant density centrifugation of isolated DNA and estimation of the proportions of Form I and Form Ic DNA.

The superhelicity of radioactively labeled closed circular DNA was analyzed by equilibrium centrifugation in CsCl gradients containing propidium diiodide (CsCl-PDI) (Hudson *et al.*, 1969). It was important here to be sure of including all but only the monomeric DNA (9-24s) for the analyses because oligomers of polyoma DNA will band at a similar position as Form Ic (Bourgaux, 1973). DNA preparations for CsCl-PDI analyses in this work did not contain any oligomeric forms.

Aliquots of ethanol precipitated viral DNA were mixed with a marker DNA labeled with an isotope different than that used for the experimental DNA in a final volume of 3.5 ml of a CsCl solution in PDI buffer (Bourgaux & Bourgaux-Ramoisy, 1972a, and a final concentration of 500 ug PDI/ml. These solutions were centrifuged to equilibrium at 20°C in a Spinco SW 27.1 or SW 40 rotor for 90 hours at 22,000 rpm. The starting density was 1.52 g CsCl/ml except the centrifugations with the SW 40 rotor which have a starting density of 1.58 g CsCl/ml.

The following procedure was used to estimate the proportions of Form I and Form Ic DNA in a composite curve generated from a PDI buoyant density gradient (for example, Fig. 20(b)): From the non-overlapping region of the ^{14}C-marker DNA (i.e. the lighter density half of the Gaussian distribution. e.g. fractions 56 to 66 of Figure 20(b)) a series of paired points, one from the marker DNA and one from the test DNA, were selected which possess a constant ratio between the experimental and marker radioactivities (Experimental CPM/Marker CPM). This ratio was used to fit the profile of marker DNA under that of the composite DNA, thereby defining the area of the experimental curve comprised by the Form I component. The remaining area under the composite curve indicates the proportion of Form Ic.

Reconstruction experiments indicate that the possible error by employing this method of quantitation is less than 9.5%.

4. Benzoylated-naphthoylated DEAE (BND)-cellulose chromatography of isolated radioactively labeled DNA.

Replicative intermediate polyoma DNA (RI) was prepared by chromatography on BND-cellulose columns according to Kiger and Sinsheimer (1969) as previously described (Sadoff & Cheevers, 1973).

(VIII) PREPARATION OF RADIOACTIVELY LABELED MARKER DNA

^{14}C-form I DNA marker was prepared by labeling infected cultures with Thymidine-2-^{14}C (0.2-0.5 μCi/ml) from 10-30 hours post-infection. When a ^{3}H-form Ic DNA marker was required, infected cultures were labeled with ^{3}H-TdR (150 μCi/ml) at 28-34h post-infection. Ten μg/ml of cycloheximide was present in such preparations from 27 hour post-infection until the cells were ready for DNA extraction and primary isolation by neutral sucrose velocity sedimentation.

(IX) MEASUREMENT OF RADIOACTIVITY

Sucrose gradients were collected using an ISCO model 640 fractionator equipped with a model UA-2 ultraviolet analyzer at 254 nm. PDI-CsCl gradients were collected using a Buchler Instruments fraction collector. Three-drop fractions were collected with a constant positive pressure from the top generated by a multispeed transmission (Harvard Apparatus Co., Dover, Mass., U.S.A.) pushing a column of paraffin oil at a rate of 0.408 ml/min. Fractions from gradients or BND-cellulose columns were mixed with an equal volume of cold 10% trichloroacetic acid (TCA), and the acid-insoluble fraction collected onto Millipore or Gelman cellulose ester filters (0.45 μm pore size). The filters were washed with 5% TCA in 95% ethanol and counted in toluene scintillation fluid as previously

described (Cheevers, Kowalski & Yu, 1972). Alternatively, fractions were mixed with 2 drops of an aqueous solution of fraction V bovine serum albumin (1 mg/ml) and precipitated with an equal volume of 10% TCA. The acid-insoluble fraction was collected by filtration onto Whatman GF/C glass fiber papers and processed for scintillation counting as described above.

RESULTS*

*A portion of this thesis has already been published or submitted to be published as papers (Cheevers *et al.* 1972, Yu *et al.* 1975a, Yu & Cheevers 1975a, Yu & Cheevers 1975b) or in abstract form (Cheevers *et al.* 1971, Yu *et al.* 1975b).

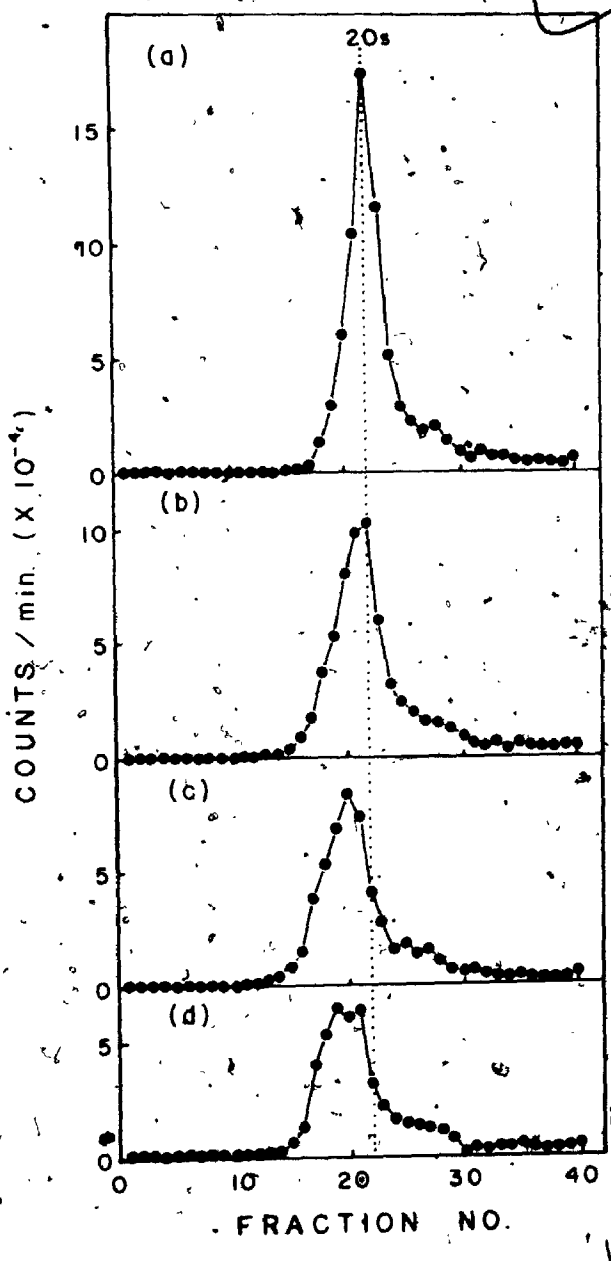
PART I

EFFECT OF A *DE NOVO* PROTEIN SYNTHESIS BLOCK ON THE INITIATION OF POLYOMA DNA.

1. Effect of Cycloheximide on the Synthesis of Polyoma Virus DNA

Cycloheximide, at a concentration of 10 µg/ml, blocks the cumulative synthesis of protein within 1-2 minutes in polyoma virus-infected cells (See Fig. 23, Part III of this section). The experiment described in Fig. 4 shows the effect of inhibition of protein synthesis on the formation of viral DNA. In untreated cells pulse-labeled for 1 hour with ^{3}H-TdR, newly-synthesized viral DNA was comprised predominantly of closed-circular form I molecules, sedimenting at 20S in neutral solution (Fig. 4(a)). Some residual RI, sedimenting at ∿25S, was also apparent. However, quantitation of this component is not possible because it partially sediments in the region of 20S DNA (Cheevers, Kowalski & Yu, 1972; Sadoff & Cheevers, 1973) and because of the presence of small amounts of fragmented cellular DNA (Sadoff & Cheevers, 1973) and dimeric viral DNA (Meinke & Goldstein, 1971) in this region of the gradients. Two main points are evident from the sedimentation profiles of viral DNA from cycloheximide-treated cells (Fig. 4(b), (c), (d)). (i) With increasing time of pre-treatment of cells with cycloheximide, the rate of ^{3}H-TdR incorporation into viral DNA was progressively reduced. After 60 minutes pre-treatment (Fig. 4(d)), the rate of

Fig. 4. Velocity sedimentation in neutral sucrose gradients of viral DNA synthesized in untreated and cycloheximide-treated polyoma infected cells. Infected cells were incubated for various periods of time with medium or medium containing 10 µg/ml cycloheximide. The cultures were then pulse-labeled with ^{3}H-TdR (50 µCi/ml) (27-28 hours post-infection). Crude SDS-lysates of cells were prepared as described in MATERIALS AND METHODS and sedimented in neutral sucrose gradients (SW 27.1 rotor, 22000 rpm, 14 hours, 23°C). High-molecular-weight cellular DNA, which sedimented to the bottom of the gradient, is not shown. (a) Untreated cells. (b) 10 minute pre-treatment with cycloheximide. (c) 30 minute pre-treatment with cycloheximide. (d) 60 minute pre-treatment with cycloheximide. The direction of sedimentation in all gradients is from left to right.


(a)
20s
(b)
(c)
(d)
COUNTS / min. (X 10⁻⁴)
FRACTION NO.
0
5
10
15
20
30
40

viral DNA synthesis at 27-28 hours post-infection was about 30% of that in untreated cells (Fig. 4(a)). The kinetics of inhibition are "step-down", *i.e.* a rapid initial decay, occurring over a period of about 10-15 minutes (See Fig. 23 in Part III of this section), reduces the rate of DNA synthesis by nearly 50%. Thereafter, synthesis proceeds with a linear but slowly declining rate. (ii) The second point to be made from Fig. 4(b-d) is that newly-synthesized 20S viral DNA was gradually replaced by material sedimenting at 13-18S. This component, which I have termed form I_c is a monomeric closed-circular viral DNA species deficient in super-helical turns. It is formed in polyoma-infected cells treated with puromycin (Bourgaux & Bourgaux-Ramoisy, 1972) or cycloheximide (Cheevers, 1973). Detailed information on form Ic DNA, is in Part II & III of this section.

2. Mechanism of Inhibition of Viral DNA Synthesis in Cycloheximide-Treated Cells

The inhibition of polyoma DNA synthesis by cycloheximide could occur at several molecular levels. In this work, I have considered the following: (i) initiation of new rounds of genome replication, (ii) nascent DNA strand growth within the RI population, and (iii) maturation of RI into closed-circular progeny DNA.

3. Effect of cycloheximide on the rate of synthesis of closed-circular DNA and viral RI.

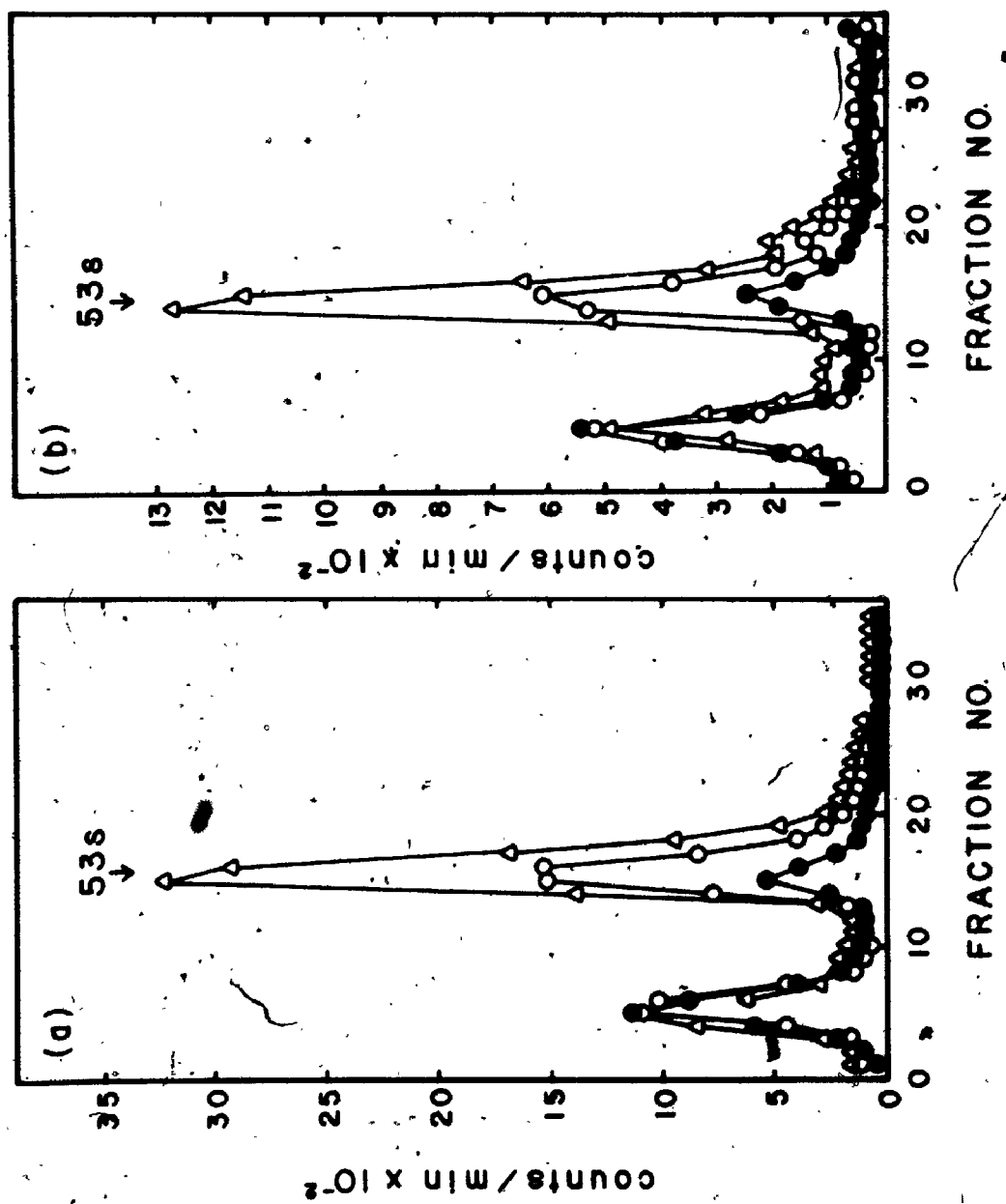
This study compares the relative rate of incorporation of ^{3}H-TdR into closed-circular DNA and viral RI in cycloheximide-treated cells. The rationale is as follows: If cycloheximide acts exclusively at the level of initiation of viral DNA or if initiation and the subsequent processing of RI are affected equally, the rate of inhibition of the synthesis of total viral DNA (closed-circular + RI) should parallel the inhibition of RI synthesis alone. Alternatively, if the rate of progression or maturation of replicating DNA molecules is slow as compared to the initiation of new molecules, labeled RI will accumulate relative to the total DNA synthesized.

(a) Effect of Cycloheximide on the cumulative synthesis of total viral DNA and RI molecules.

In the first experiment, the cumulative synthesis of total viral DNA and RI was compared in control and cycloheximide-treated cultures. Cells were treated for 20 minutes with medium or medium containing cycloheximide and then labeled with ^{3}H-TdR. Viral DNA was isolated at 15-minute intervals over a total labeling period of 1 hour by velocity sedimentation in neutral sucrose and then further resolved into closed-circular

and RI components by sedimentation in alkaline sucrose. Under these conditions, component I polyoma DNA (Cheevers, Kowalski & Yu, 1972; Vinograd *et al.*, 1965) and component I_c (Bourgaux & Bourgaux-Ramoisy, 1972; Cheevers, 1973) sediment at 53S, because their closed-circular conformation renders them resistant to alkali-induced strand separation. In contrast, nascent RI, which has single-strand interruptions at the growing points (Bourgaux & Bourgaux-Ramoisy, 1971) are susceptible to alkaline denaturation and sediment near the top of the gradient. Figure 5(a) shows representative sedimentation profiles of DNA labeled for 15, 30, and 60 minutes in untreated cells. The analogous profiles of DNA from drug-treated cells are shown in Fig. 5(b). Note that there was an obvious increase with time of labeled 53S DNA in both untreated and drug-treated cultures, whereas labeled RI did not appear to accumulate in either case. Figure 6 shows the cumulative synthesis of viral DNA from two experiments. In untreated cells, ^{3}H-TdR was incorporated linearly into viral DNA. The RI population was labeled with steady-state kinetics, consistent with an average turnover time for replicating molecules of 15 minutes or less. Bourgaux and Bourgaux-Ramoisy (1971) have estimated that the duplication time of polyoma DNA at 37°C is close to 4 minutes. Cheevers (unpublished data) also arrived at a similar estimate using saturation labeling of RI with ^{3}H-TdR. The cumulative incorporation

Fig. 5. Representative alkaline sedimentation profiles of viral DNA synthesized in untreated and cycloheximide-treated polyoma infected cells. Infected cells were incubated for 20 minutes with medium or medium containing cycloheximide. ^{3}H-TdR (20 μCi/ml) was then added (24 hours post-infection). At various times thereafter, viral DNA was isolated by sedimentation in neutral sucrose gradients (SW 27 rotor, 24000 rpm, 12 hours, 23°C), concentrated by ethanol precipitation, and dissolved in 0.1 x SSC. The viral DNA was then alkali-denatured and resolved into 53s closed-circular and RI components by velocity sedimentation analysis in alkaline sucrose gradients (SW 27 rotor, 26500 rpm, 8 hours, 23°C). (a) Untreated cells. (b) Cycloheximide-treated cells. (● - ●) 15 minute labeling period. (○ - ○) 30 minute labeling period. (Δ - Δ) 60 minute labeling period.


(a)
536
counts / min x 10⁻²
FRACTION NO.
(b)
538
counts / min x 10⁻²
FRACTION NO.

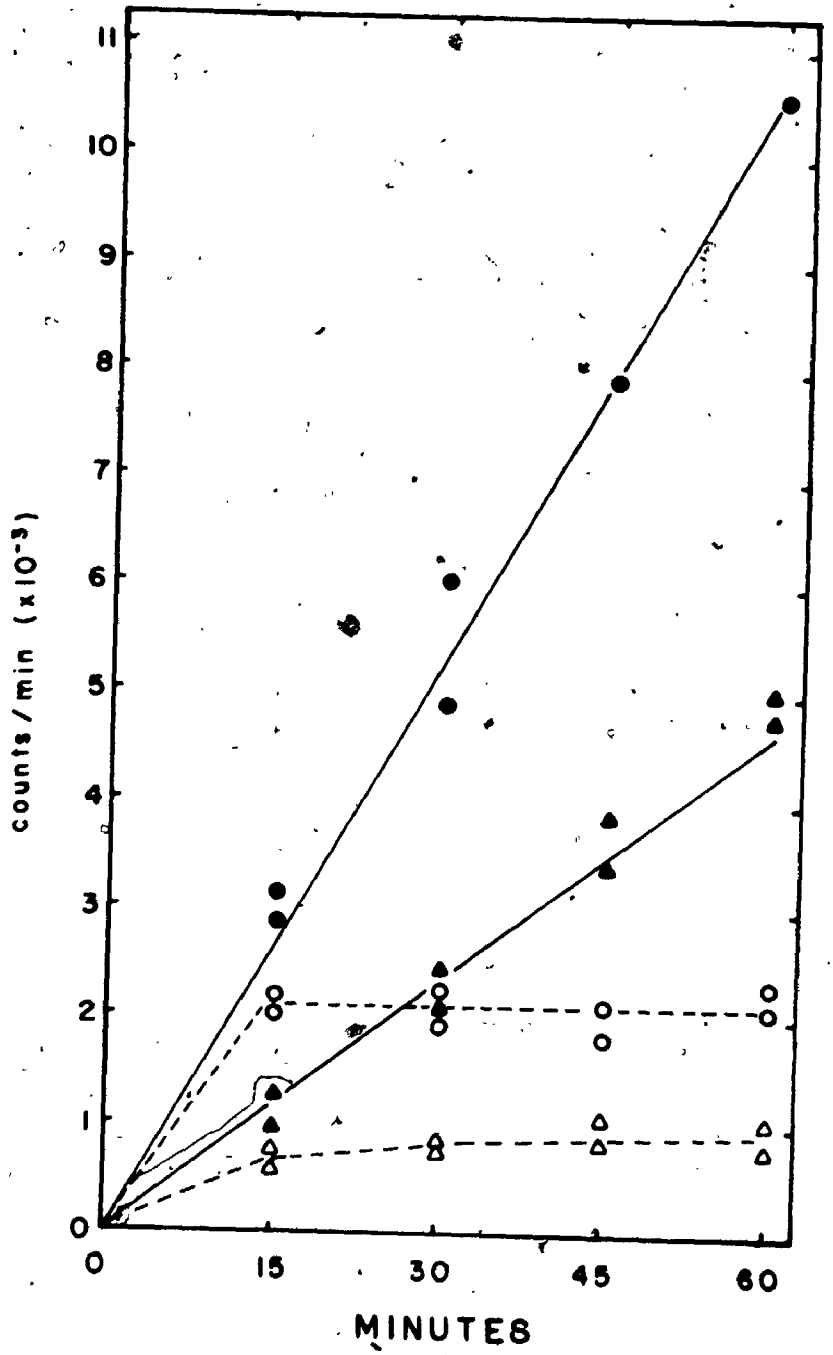
of label into viral DNA in cycloheximide-treated cells was also linear, as expected the rate of accumulation of labeled DNA was only about 40% of that in untreated cells. Labeling of the RI component was also steady-state, reaching equilibrium at the same time as in control cells, but indicating a much smaller RI population.

The results of Fig. 5 and 6 indicate that cycloheximide causes a reduction in the size of the replicating pool of viral DNA by more than 50%, whereas the rate of turnover of replicating molecules is not affected. This suggests that inhibition of protein synthesis results in either an exclusive decay of the initiation of new rounds of viral DNA synthesis or that this process and the maturation of RI into closed-circular DNA are affected equally. This point is substantiated by the experiment shown in the next section.

(b) Effect of various durations of cycloheximide pretreatment on the synthesis of total viral DNA and RI molecules.

In this study (see Fig. 7) infected cells were pre-treated with cycloheximide for 20-120 minutes and then labeled with ^{3}H-TdR for 1 hour. In untreated cells, RI constituted about one-tenth of labeled viral DNA. With increasing periods of cycloheximide pre-treatment, the

Fig. 6. Cumulative synthesis of total polyoma DNA and RI in cycloheximide-treated cells. Data were derived according to the procedures described in Fig. 5. (●-●) Total viral DNA, untreated cells. (▲-▲) Total viral DNA, cycloheximide-treated cells. (O--O) RI, untreated cells. (Δ--Δ) RI, cycloheximide-treated cells.


counts / min (x10⁻³)
0
1
2
3
4
5
6
7
8
9
10
11
0
15
30
45
60
MINUTES

amount of viral DNA synthesized was progressively reduced; by 2 hours, only about 20% as much viral DNA was labeled as in the untreated cells. However, the proportion of labeled RI remained at approximately 10-11% of the viral DNA.

4. Effect of cycloheximide on the rate of conversion of viral RI into progeny closed-circular DNA.

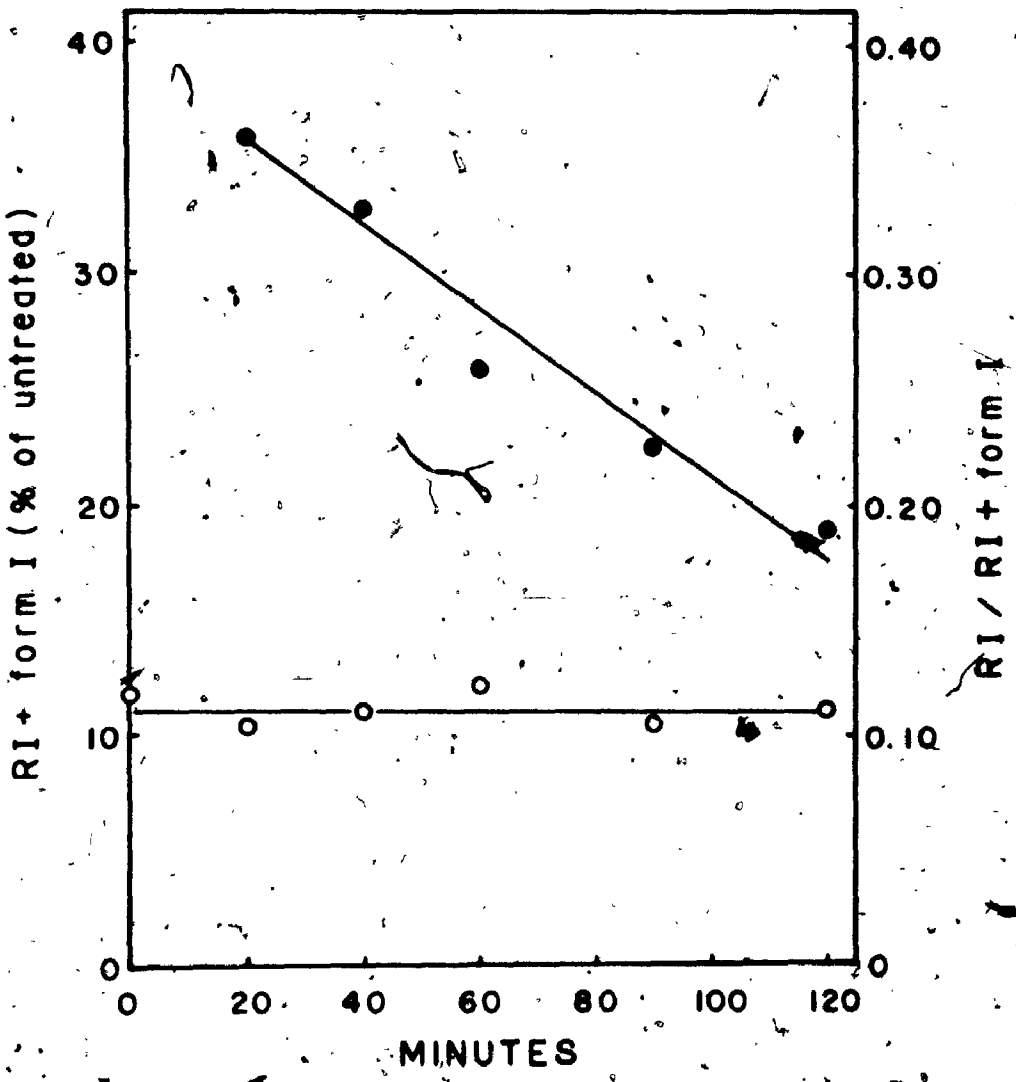
The results of Fig. 5-7 appear to rule out the possibility that the maturation of polyoma RI DNA is suppressed in the absence of protein synthesis to a greater or lesser degree than the initiation of new rounds of replication. It is significant to point out that were this the case, the experiments would have detected it. This is evident because Cheevers, Kowalski and Elliott (unpublished data) used an identical experimental approach to show that the initiation of new rounds of viral DNA synthesis and the maturation of viral RI are inhibited at differential rates in polyoma-infected cells in which RNA synthesis has been blocked. In addition Manteuil and Girard (1974) successfully used a similar method to analyze the specific effects of various inhibitors of DNA synthesis on SV 40 DNA replication. Thus, I conclude that the mechanism of inhibition of polyoma DNA synthesis by cycloheximide is either at the level of initiation of new rounds of genome replication

or at this level plus an equal effect on the rate of maturation of replicating molecules. To differentiate between these possibilities, the conversion of labeled RI into closed-circular DNA was examined in pulse-chase analyses.

Infected cells were incubated with medium or medium containing cycloheximide for various periods of time. The cultures were then shifted to 25°C to retard the rate of DNA synthesis, and ^{3}H-TdR was added to pulse-label replicating DNA. Radioactive medium was then replaced with medium containing unlabeled TdR, and the cultures were incubated further for various periods. After each period of pulse-labeling or chase, viral DNA was isolated by neutral sucrose gradient centrifugation. The viral DNA was concentrated and further fractionated by sedimentation in alkaline sucrose gradients or by BND-cellulose chromatography.

Table 1 shows the chromatographic properties on BND-cellulose of viral DNA pulse-labeled for 1.5 minutes and chased for various periods of time in control and cycloheximide-treated cells. RI molecules are selectively eluted from BND-cellulose in 1 M NaCl-2% caffeine (Levine, Kang & Billheimer, 1970; Sadoff & Cheevers, 1973). Pulse-labeled DNA in untreated cells was comprised of 83% RI. This proportion decreased with

Fig. 7. Effect of cycloheximide on the rate of synthesis of total polyoma DNA and RI. Infected cells were incubated with medium containing cycloheximide for the indicated times and then labeled with ^{3}H-TdR (20 µCi/ml) at 24 to 25 hours post-infection. Viral DNA was isolated and resolved into 53S and RI components as described in Fig. 5. Inhibition of total viral DNA synthesis (● - ●) is expressed as ^{3}H-TdR incorporated into 53S + RI DNA in cycloheximide-treated cells/untreated cells x 100. The proportion of RI DNA (O - O) was estimated from sedimentation profiles of viral DNA in alkaline sucrose gradients (see Fig. 5).


40
30
20
10
0
0.40
0.30
0.20
0.10
0
0
20
40
60
80
100
120
MINUTES
RI + form I (% of untreated)
RI / RI + form I

LEGEND: TABLE 1

[a]Polyoma-infected cells, actively synthesizing viral DNA, were shifted from 37°C to 25°C. After 10 minutes at 25°C, the cultures were treated with medium or medium containing 10 µg/ml cycloheximide. After 40, 75, or 120 minutes, the cultures were pulse-labeled with ^{3}H-TdR (100 µCi/ml). Pre-treatment times were arranged so that pulse-labeling was always done at 25 hours post-infection. Some cultures were harvested by trypsinization immediately after the pulse-labeling period. The remaining cultures were incubated with medium containing unlabeled TdR with or without cycloheximide for various indicated times and then harvested. Viral DNA was isolated by velocity sedimentation in neutral sucrose gradients as described in Fig. 4, concentrated by ethanol precipitation, and dissolved in 0.3 M NaCl - 0.001 M EDTA - 0.01 M Tris, pH 8.1. This solution was then chromatographed on BND - cellulose columns as previously described (Sadoff & Cheevers, 1973).

[b]Prior to BND - cellulose chromatography, aliquots of these DNA preparations were denatured by addition of 0.1 volume of 1 N NaOH - 0.01 M EDTA analyzed by velocity sedimentation in alkaline sucrose gradients (see Fig. 8).

[c]Viral RI DNA, eluted from BND - cellulose in the

TABLE 1

PULSE - CHASE ANALYSIS OF REPLICATING POLYOMA DNA BY BND - CELLULOSE CHROMATOGRAPHY[a]

cycloheximide pre-treatment (minutes)	^{3}H-TdR pulse (minutes)	unlabeled TdR plus cycloheximide	chase (minutes) minus cycloheximide	counts/minute eluted in caffeine total eluted from BND - cellulose			
				I*	II*	III*	Avg.
0	1.5		0	0.78[c]	0.85[c]	0.85[b]	0.83
"	"		5	0.79[c]			0.79
"	"		8	0.63	0.69[c]	0.59[b]	0.64
"	"		10	0.62[c]			0.62
"	"		15	0.50	0.60[c]	0.43	0.51
"	"		20	0.38[c]			0.38
"	"		25	0.33	0.24[c]	0.24[b]	0.27
40	1.5	0		0.75[c]	0.78		0.77
"	"	8		0.63[c]			0.63
"	"	15		0.43[c]			0.43
"	"	25		0.26[c]			0.26
75	1.5	0		0.82[bc]	0.79		0.81
"	"	8		0.67[bc]			0.67
"	"	15		0.36[b]			0.36
"	"	25		0.18[bc]			0.18
120	1.5	0		0.79	0.74		0.77
"	"	10		0.46	0.65		0.56

*. different experiments or replicate samples.

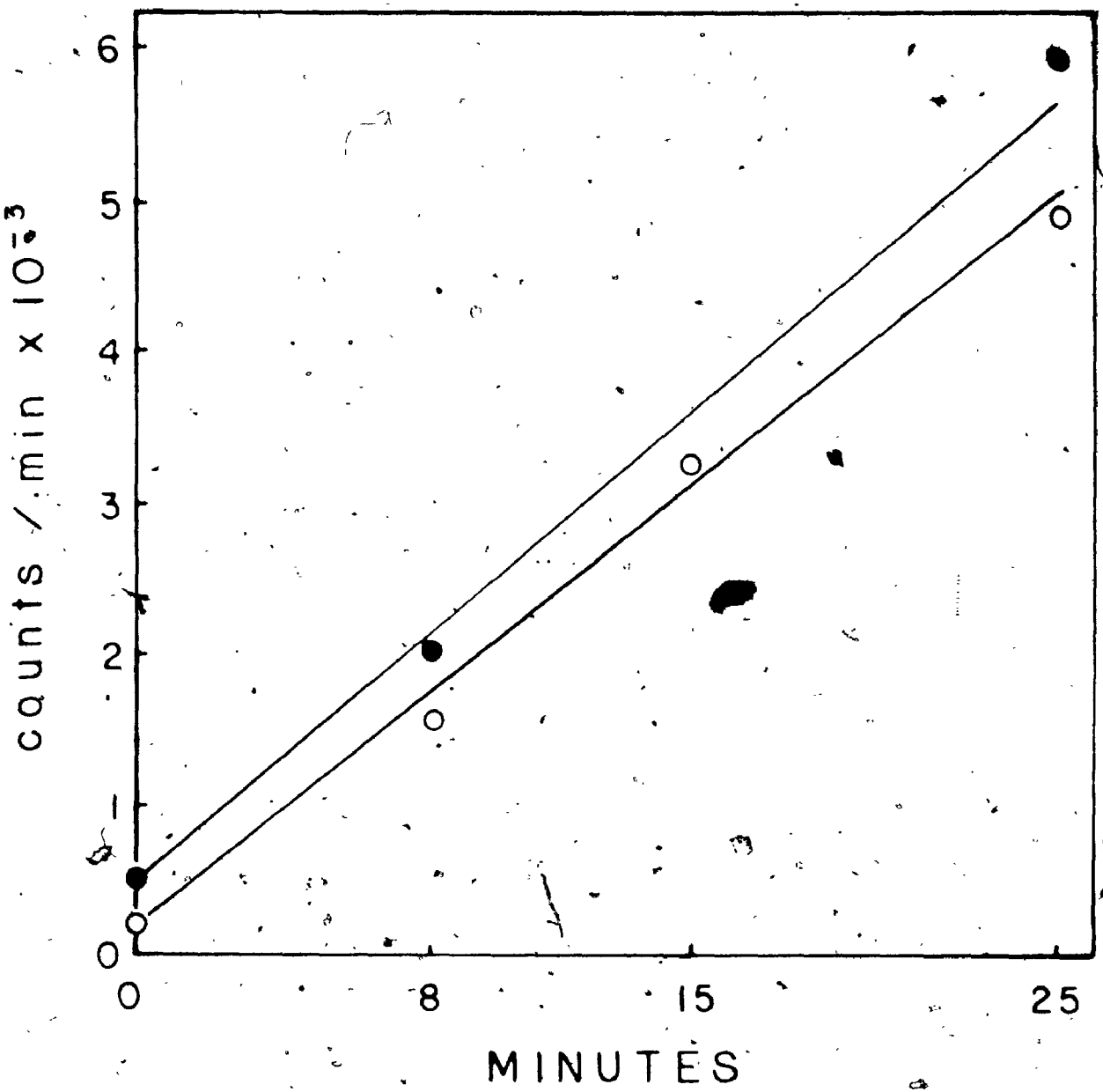
1 M NaCl - 2% caffeine fraction, was concentrated by ethanol precipitation and denatured by addition of 1 N NaOH - 0.01 M EDTA. The size distribution of nascent DNA strands was then determined by sedimentation analysis at alkaline pH as described in MATERIALS AND METHODS (see Fig. 9).

increasing chase periods, so that, by 25 minutes, these molecules comprised only about one-fourth of the viral DNA. Pre-treatment of cells with cycloheximide for 40, 74, or 120 minutes did not change the proportion of RI in pulse-labeled DNA. In addition, the presence of cycloheximide during the chase did not alter the rate of disappearance of viral RI.

DNA preparations marked (b) in Table 1 were analyzed prior to BND-cellulose chromatography by sedimentation in alkaline sucrose to determine the proportion of 53S superhelical DNA (Fig. 8). In untreated cells, 53S viral DNA accumulated linearly during the 25-minute chase period. 53S DNA formed during the 1.5-minute pulse-labeled was reduced as a result of pre-treatment of cells with cycloheximide; however, the presence of cycloheximide during the chase did not alter the rate of accumulation of closed-circular DNA (form $I + I_c$).

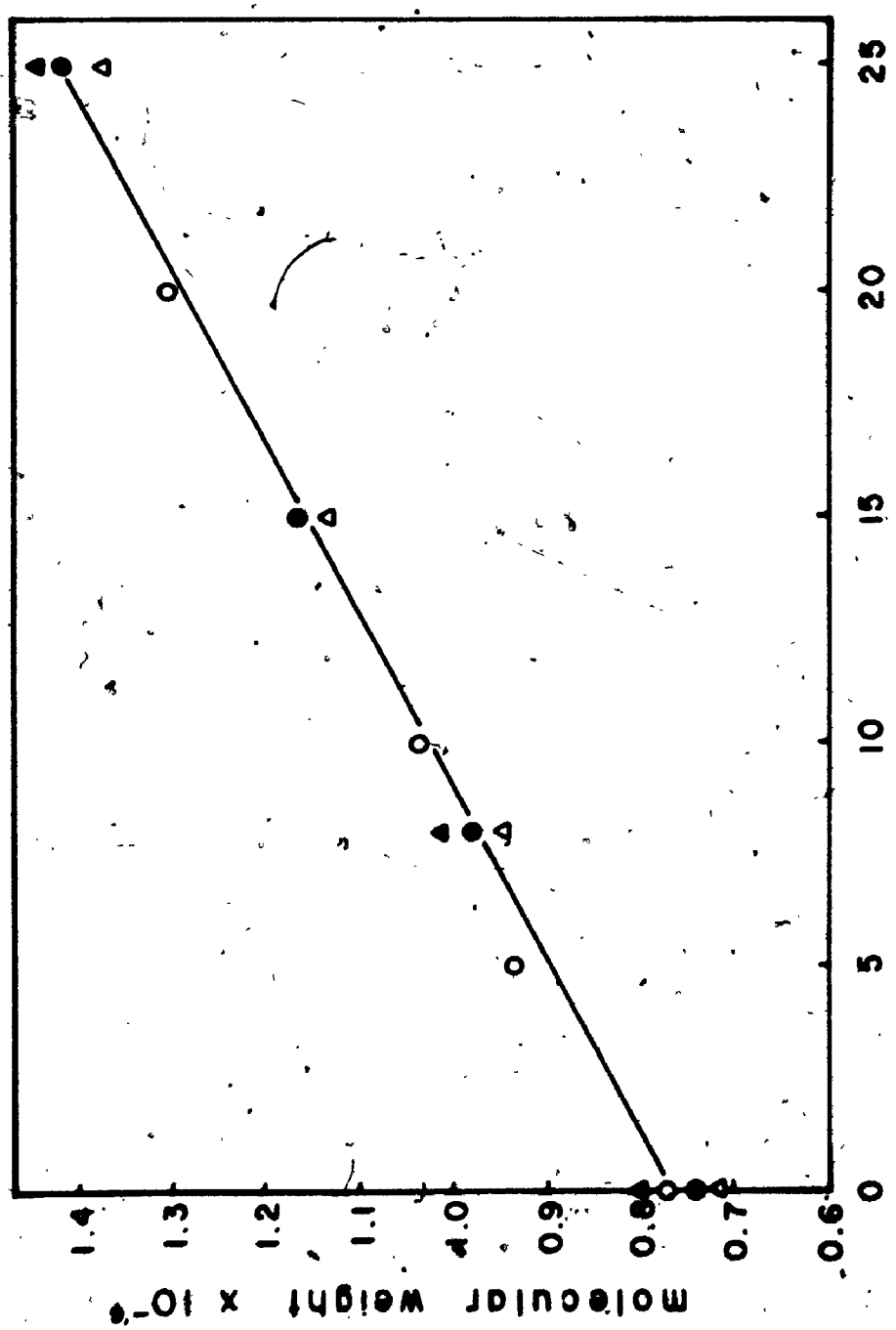
The size distribution of nascent strands of RI molecules, prepared by BND-cellulose chromatography (DNA samples marked (c) in Table 1), was analyzed by sedimentation in alkaline sucrose gradients. The results (Fig. 9) show that pulse-labeled nascent RI strands had an average molecular weight of approximately 7.5×10^5 daltons. With increasing chase time, these strands were elongated at a constant rate of 2.7×10^4 daltons per minute, attaining, by 25 minutes, a molecular weight of 90-95% of unit genome

Fig. 8. Accumulation of 53S closed-circular DNA during an unlabeled TdR chase of pulse-labeled polyoma RI. DNA preparations marked (b) in Table 1 were alkali-denatured and sedimented in alkaline sucrose gradients as described in Fig. 5. (● - ●) 53S DNA from untreated cells pulse-labeled 1.5 minutes with ^{3}H-TdR and chased in medium containing unlabeled TdR. (○ - ○) 53S DNA from cells treated 75 minutes with cycloheximide, pulse-labeled with ^{3}H-TdR, and chased in medium containing unlabeled TdR and cycloheximide.


counts / min x 10^{-3}
0
1
2
3
4
5
6
0
8
15
25
MINUTES

2 OF/DE 3

Fig. 9. Effect of cycloheximide on nascent polyoma DNA strand elongation. BND - cellulose purified viral RI preparations marked (c) in Table 1 were concentrated by ethanol precipitation and denatured by addition of 0.1 volume of 1 N NaOH - 0.01 M EDTA. The size distribution of nascent strands was then determined by velocity sedimentation at alkaline pH as described in MATERIALS AND METHODS. (●—● O—O) Viral RI from untreated cells labeled 1.5 minutes with ^{3}H-TdR and chased in medium containing unlabeled TdR. (Δ—Δ) Viral RI from cells treated 40 minutes with cycloheximide, pulse-labeled with ^{3}H-TdR, and chased in the presence of cycloheximide. (▲—▲) Viral RI from cells treated 75 minutes with cycloheximide, pulse-labeled with ^{3}H-TdR, and chased in the presence of cycloheximide.


molecular weight x 10^{-6}
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0
5
10
15
20
25
MINUTES

size (1.5×10^6 daltons).

The results described above eliminate the possibility that cycloheximide affects the conversion of polyoma RI molecules into closed-circular progeny DNA. Therefore, I conclude that in the absence of protein synthesis, polyoma DNA synthesis is inhibited exclusively at the level of initiation of new rounds of genome replication.

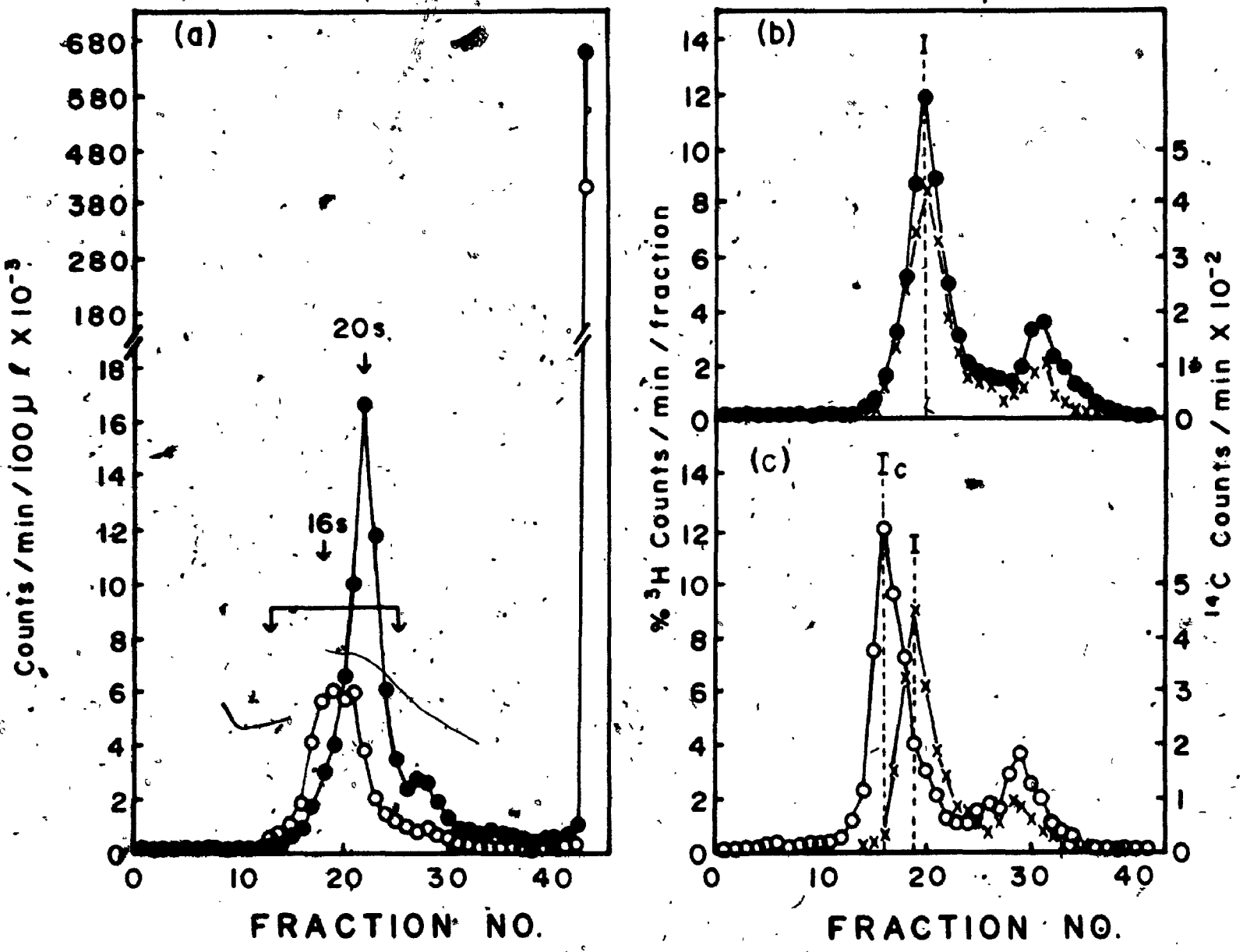
PART II

EFFECT OF A *DE NOVO* PROTEIN SYNTHESIS BLOCK ON THE SUPERHELIX-DENSITY OF POLYOMA DNA.

1. Characterization of form Ic DNA. Figure 10(a) shows the sedimentation properties at neutral pH of polyoma DNA formed in the presence of cycloheximide. Two points are evident: (i) Much less viral DNA was synthesized than in untreated cultures. This effect has been shown to result exclusively from limited initiation of new rounds of genome replication (See Part I of this section). (ii) 20s form I DNA was replaced by a DNA component sedimenting at 16s. This component is form Ic. Form Ic sediments as monomeric closed-circular viral DNA under alkaline conditions (Cheevers, 1973) and shows a decreased superhelix density by isopycnic analysis in CsCl-PDI (Fig. 10(c)) as compared to form I DNA (Fig. 10(b)).

The superhelix density of form Ic was determined as follows: Forms I and Ic, labeled with [^{14}C] TdR and [^{3}H] TdR respectively, were isolated as described in Fig. 10(a) and purified by CsCl-PDI centrifugation followed by Sephadex G-50 column chromatography to remove CsCl and PDI. After concentration by ethanol precipitation, purified forms I and Ic were mixed, partially converted to form II by limited digestion with deoxyribonuclease (Vinograd *et al.*, 1965) and centrifuged to equilibrium in CsCl-PDI. The distance between the bands corresponding to ^{3}H-II and ^{3}H-Ic was compared with that of ^{14}C-II and ^{14}C-I according to the Eason and Vinograd (1971) equation:

Fig. 10. Sedimentation and dye-buoyant density centrifugation properties of polyoma DNA synthesized in cycloheximide-treated cells. (a) Velocity sedimentation at neutral pH. Infected cells were pretreated for 90 minutes at 28 hours post-infection with medium with or without cycloheximide (10 µg/ml) and then pulse-labeled for 1 hour with [^{3}H] TdR (25 µCi/ml). Cell lysates were prepared by treatment with sodium dodecyl sulfate and centrifuged in neutral sucrose gradients as described in Materials and Methods. (● - ●) Untreated cells. (O - O) Cycloheximide-treated cells. The direction of sedimentation is from left to right in all velocity sedimentation analyses. The indicated fractions in (a) were combined, the DNA was concentrated by ethanol precipitation, and analyzed by isopycnic centrifugation in CsCl-PDI (rotor SW 50.1) : (b) Untreated cells. (● - ●) [^{3}H] DNA; (x-x) [^{14}C] marker form I DNA. (c) Cycloheximide treated cells. (O - O) [^{3}H] DNA; (x-x) [^{14}C] marker form I DNA. Density increases from right to left in all CsCl-PDI gradients.


(a)
(b)
(c)
Counts / min / 100 µl X 10⁻³
% ³H Counts / min / fraction
¹⁴C Counts / min X 10⁻²
20s
16s
I
Ic
FRACTION NO.
FRACTION NO.

$$\Delta \sigma_o = 0.1 \left[(\Delta r/\Delta r^*) - 1 \right]$$

My measurements indicate a shift in superhelix density from I to Ic equivalent to $\Delta\sigma_o = 0.0195$, which corresponds to a decrease in the number of superhelical turns of about two-thirds (Keller & Wendel, 1974; Vinograd, Lebowitz & Watson, 1968).

2. Origin of form Ic DNA. The purpose of this experiment was to test the possibility that form Ic arises from form I molecules. Replicating form I DNA was labeled for 2 hours with [^{3}H] TdR. The fate of these labeled molecules was then determined during a chase for various periods of time with unlabeled TdR in the presence or absence of cycloheximide.

Figure 11 shows the CsCl-PDI density gradient profiles of viral DNA chased for 4 hours. Without cycloheximide (Fig. 11(a)), ^{3}H-DNA banded with marker ^{14}C-labeled form I as expected. In the absence of protein synthesis, however, a portion of ^{3}H-labeled form I DNA was chased into the position of Ic (Fig. 11(b)). The quantitative transfer of label from I to Ic during the 4-hour chase period in the presence of cycloheximide is shown in Fig. 12(b) . These data show that about one-third of form I DNA molecules replicated during a 2-hour period were diverted to the formation of component Ic during a subsequent 2-hour period in the absence of protein

Fig. 11. Representative CsCl-PDI gradients of pulse-labeled form I DNA chased in the presence or absence of cycloheximide. Infected cells were labeled with [^{3}H] TdR (150 μCi/ml) at 30 3/4 - 32 3/4 hours post-infection. Radioactive medium was then replaced with medium containing unlabeled TdR with or without cycloheximide, and incubation was continued for 4 hours. Viral DNA was isolated by neutral sucrose gradient sedimentation as described in Fig. 10 (a) and analyzed by CsCl-PDI isopycnic centrifugation (rotor SW 40). (a) Chase in the absence of cycloheximide. (b) Chase in the presence of cycloheximide. (● - ●) [^{3}H] DNA. (O - O) [^{14}C] form I DNA marker.

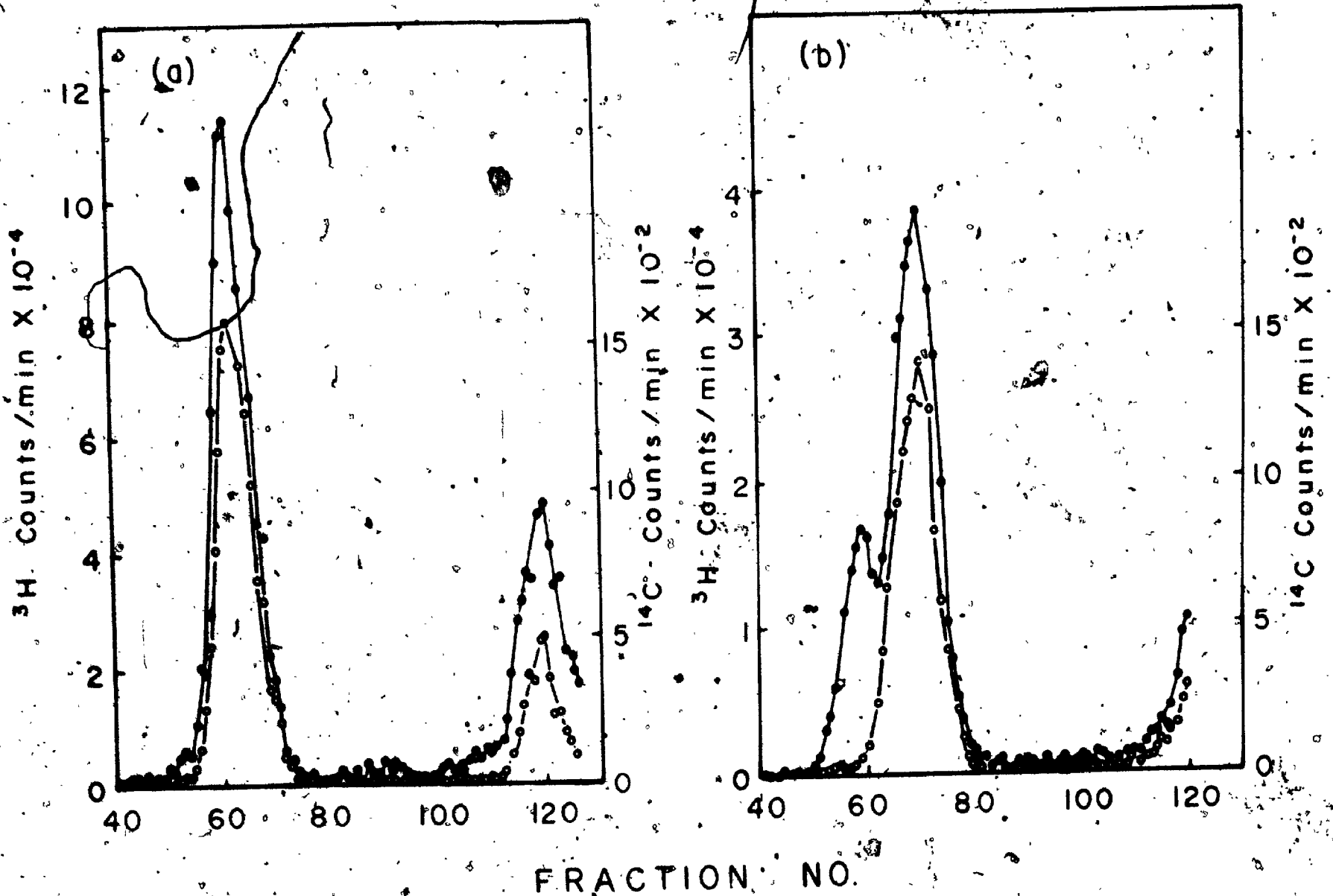
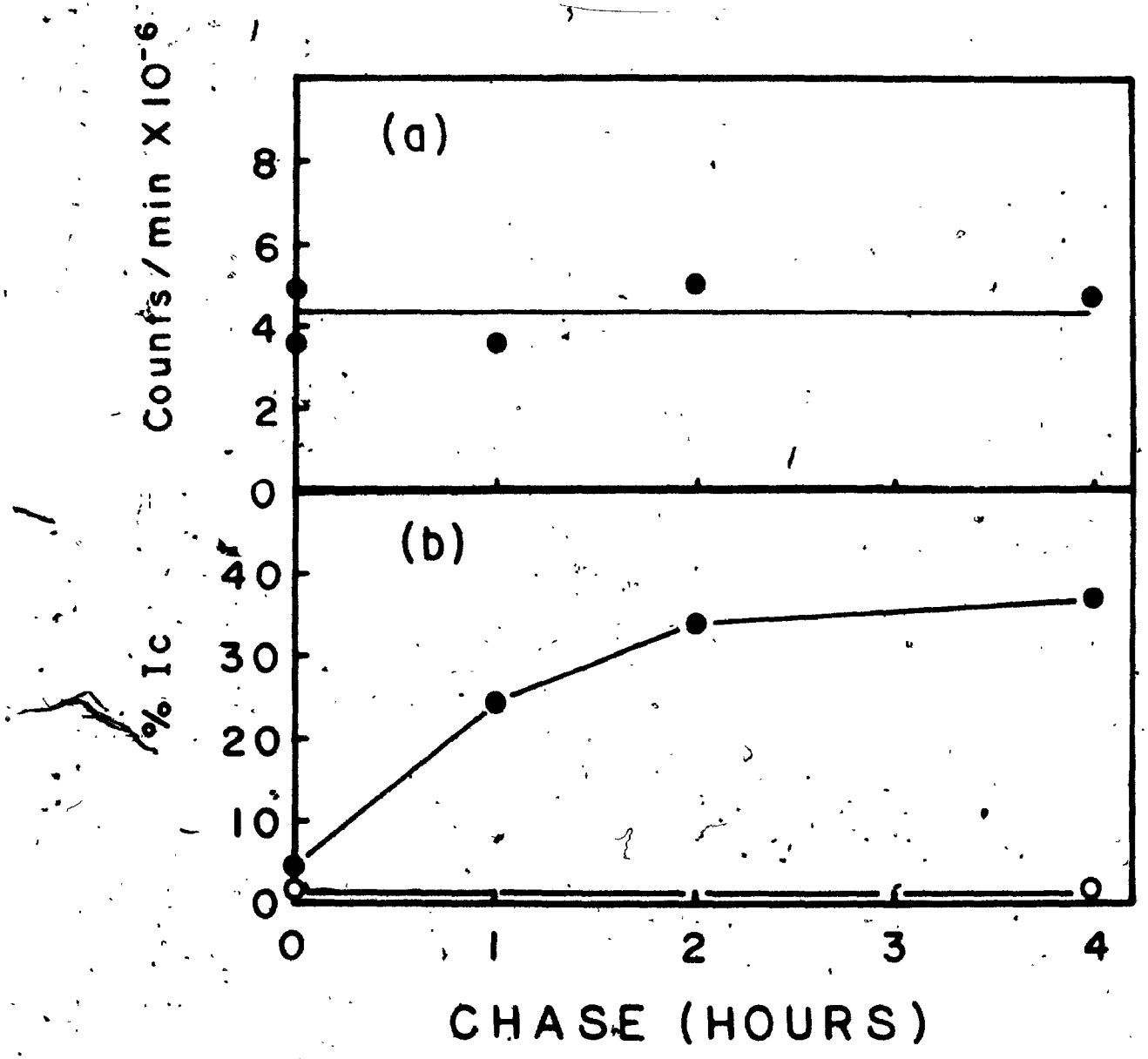

(a)
(b)
^3H Counts / min X 10^{-4}
^{14}C Counts / min X 10^{-2}
FRACTION NO.

Fig. 12. Kinetics of conversion of pre-labeled form I DNA to form Ic in the absence of protein synthesis. (a) Inhibition of [^{3}H] TdR incorporation into DNA by addition of excess unlabeled TdR (2×10^{-5}M). (b) Accumulation of form Ic DNA from pre-labeled form I in cycloheximide-treated cells. Data were derived according to procedures described in Fig. 11. (● - ●) Chase in the presence of cycloheximide. (○ - ○) Chase in the absence of cycloheximide.


(a)
(b)
Counts / min X 10⁻⁶
% Ic
0
2
4
6
8
10
20
30
40
0
1
2
3
4
CHASE (HOURS)

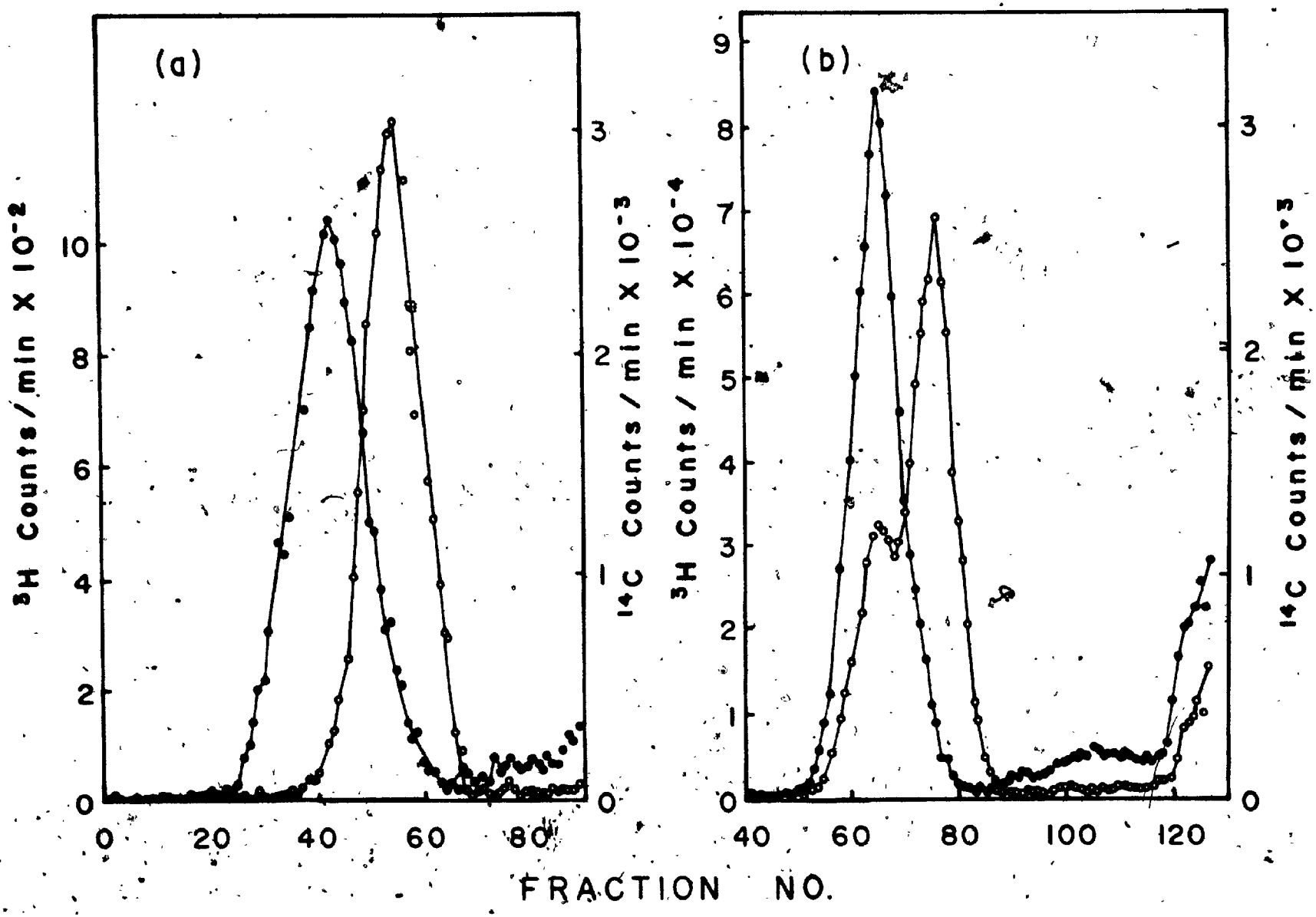
synthesis.

This finding was confirmed by the experiment described in Fig. 13. Infected cells were labeled for 2 hours with [^{14}C] TdR and then chased for an additional 2 hours with medium containing unlabeled TdR and cycloheximide. [^{3}H] TdR was administered during the last 1 1/2 hours of the chase period to label newly-formed component Ic. Figure 13(a) shows the distribution in CsCl-PDI of closed-circular ^{14}C-viral DNA before the chase period, relative to that of marker ^{3}H-form Ic. Clearly, only form I DNA was labeled. Figure 13(b) shows, however, that during the chase in the presence of cycloheximide, only form Ic DNA was made. This was accompanied by the transfer of 32% of ^{14}C-form I DNA to the position of Ic.

3. Dependency of the form I to Ic conversion on DNA replication. There are several possible explanations for the mechanism of conversion of form I DNA to form Ic. The question of whether DNA replication is involved in this process was examined as follows. Infected cells were labeled for 2 1/2 hours with [^{14}C] TdR. This was followed by a 2-hour pulse-label with [^{3}H] TdR in the presence of cycloheximide alone or in conjunction with cytosine arabinoside (ara-C).

Primary isolation of viral DNA by velocity

Fig. 13. Transfer of ^{14}C-labeled form I DNA into form Ic after a cycloheximide-induced block of protein synthesis. Infected cells were labeled with [^{14}C] TdR (2 μCi/ml) at 30 3/4 - 32 3/4 hours post-infection. Radioactive medium was then replaced with medium containing 10 μg/ml cycloheximide. Incubation was continued for 30 minutes to inhibit protein synthesis and then [^{3}H] TdR was added to a final concentration of 40 μCi/ml. Viral DNA was isolated as described in Fig. 10 (a) and analyzed by CsCl-PDI isopycnic centrifugation as described in Fig. 11. (a) ^{14}C-labeled DNA (O - O), synthesized prior to the addition of cycloheximide, centrifuged with [^{3}H] form Ic marker DNA (● - ●). (b) DNA synthesized in the presence of cycloheximide. (● - ●) [^{3}H] radioactivity. (O - O) [^{14}C] radioactivity.


(a)
(b)
^{3}H Counts / min X 10^{-2}
^{14}C Counts / min X 10^{-3}
^{3}H Counts / min X 10^{-4}
^{14}C Counts / min X 10^{-3}
FRACTION NO.

sedimentation in neutral sucrose gradients is shown in Fig. 14. ^{14}C-labeled viral DNA synthesized in the absence of cycloheximide was comprised of form I DNA sedimenting at 20S, as expected (Fig. 14(a)). ^{3}H-labeled DNA synthesized in the presence of cycloheximide sedimented in the position of form Ic (Fig. 14(b); also Fig. 10 (a)). In addition, a shoulder of labeled material appeared on the trailing edge of the ^{14}C-pre-labeled viral DNA. In the presence of ara-C (Fig. 14(c)), [^{3}H] TdR incorporation into viral DNA was drastically reduced, and the shoulder on the ^{14}C-form I DNA peak was not apparent. The residual ^{3}H-labeled DNA amounted to only 5% of the synthesis which occurred under the influence of cycloheximide but in the absence of ara-C. This material sedimented heterogeneously through the gradient rather than in an obvious peak. Analysis by CsCl-PDI equilibrium centriguation showed that this DNA was almost entirely cellular in origin.

The indicated fractions in Fig. 14 were combined, and the DNA was concentrated by ethanol precipitation and analyzed in CsCl-PDI gradients. Results are shown in Fig. 15. ^{14}C-labeled form I DNA synthesized in the absence of cycloheximide (Fig. 15(a)) was partially converted to the position of Ic when cycloheximide was administered under conditions which allowed only the synthesis of Ic (Fig. 15(b)). However, no ^{14}C-form Ic DNA was apparent in the presence of ara-C (Fig. 15(c)). These results indicate

Fig. 14. Effect of ara-C on the generation of form Ic viral DNA. Velocity sedimentation profiles in neutral sucrose gradients of: (a) Infected cells labeled with [^{14}C] TdR (3 µCi/ml) at 30 - 32 1/2 hours post-infection. (b) ^{14}C-labeled viral DNA (as in (a)) followed by a 2-hour labeling period with [^{3}H] TdR (70 µCi/ml) in the presence of 10 µg/ml cycloheximide. (c) ^{14}C-labeled viral DNA (as in (a)) followed by a 2-hour labeling period with [^{3}H] TdR (70 µCi/ml) in the presence of cycloheximide and 30 µg/ml ara-C. (O - O) ^{14}C radioactivity. (● - ●) ^{3}H radioactivity.

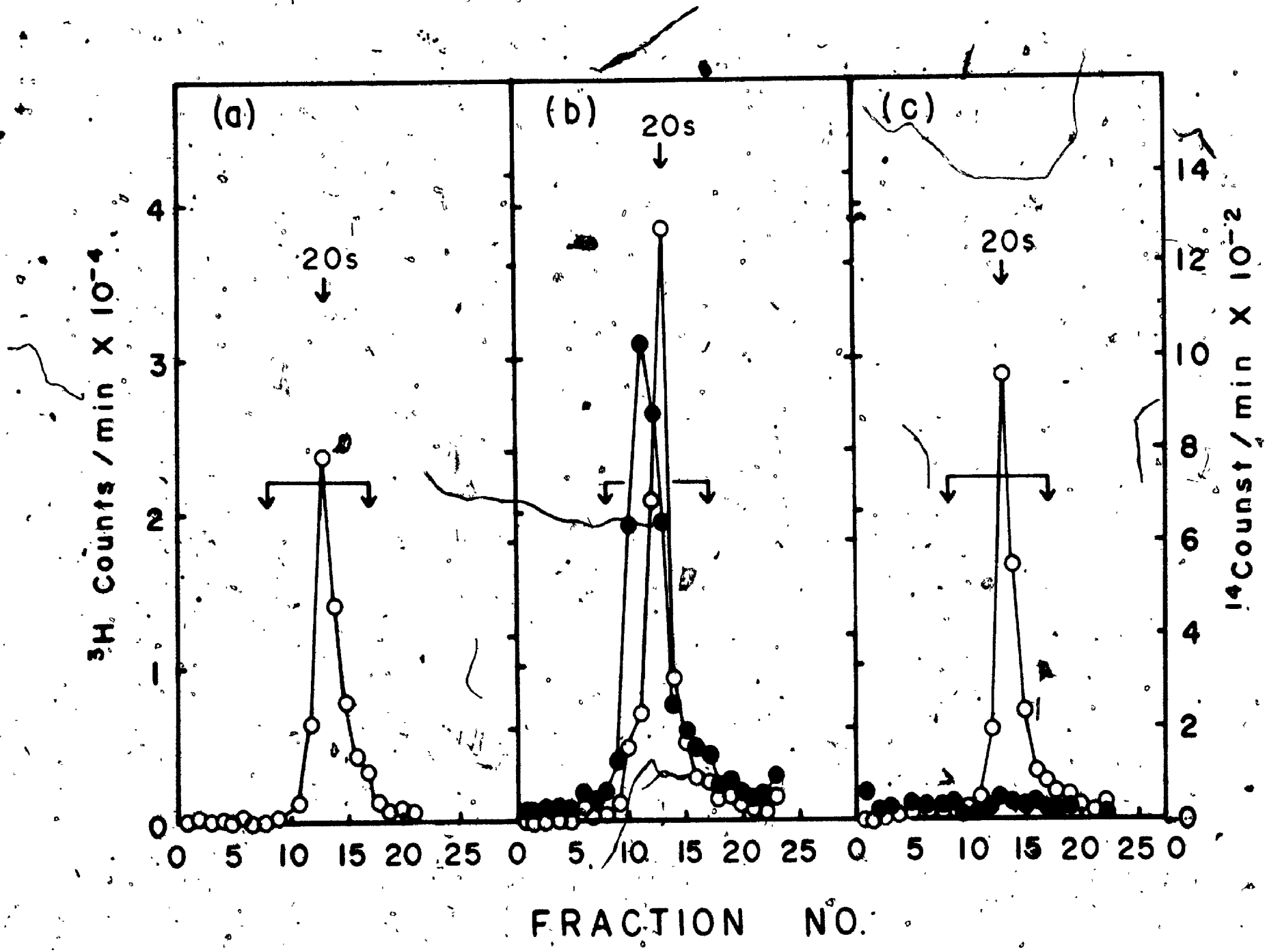
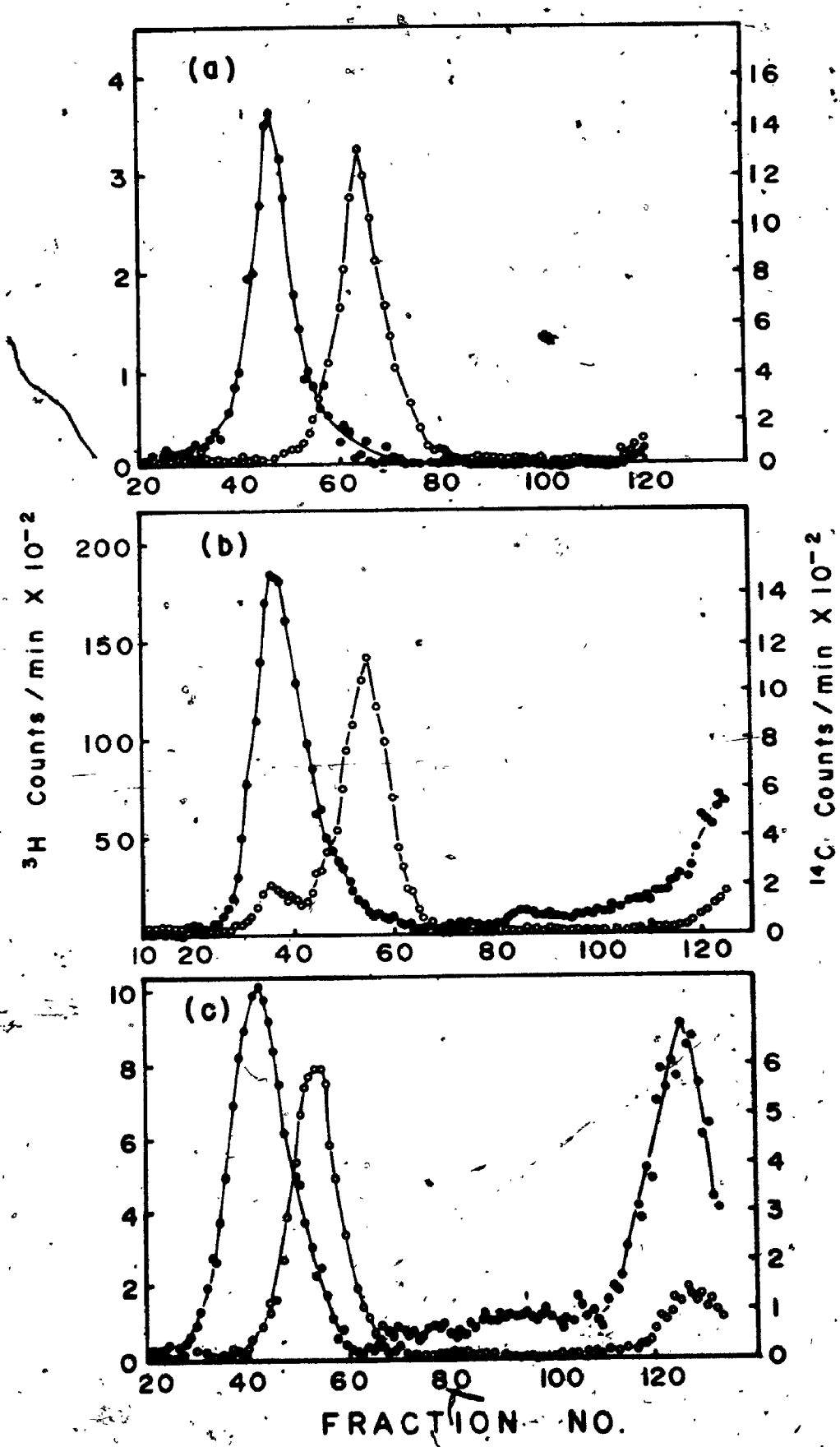

(a)
(b)
(c)
20s
20s
20s
^{3}H Counts / min X 10^{-4}
^{14}Counst / min X 10^{-2}
0
1
2
3
4
0
2
4
6
8
10
12
14
0 5 10 15 20 25 0 5 10 15 20 25 0 5 10 15 20 25 0
FRACTION NO.

Fig. 15. CsCl-PDI analysis of viral DNA formed in the presence and absence of ara-C. DNA prepared by ethanol precipitation from the neutral sucrose gradient fractions indicated in Fig. 14 was analysed by CsCl-PDI isopycnic centrifugation as described in Fig. 11. (a) ^{14}C-labeled DNA from Fig. 14 (a) centrifuged with [^{3}H] form Ic marker DNA. (b) DNA from Fig. 14 (b). (c) DNA from Fig. 14 (c) centrifuged with [^{3}H] form Ic marker DNA. (● - ●) [^{3}H] radioactivity. (O - O) ^{14}C radioactivity.


(a)
(b)
(c)
^3H Counts / min X 10^{-2}
^{14}C Counts / min X 10^{-2}
FRACTION NO.

clearly that the formation of component Ic DNA from pre-existing form I templates depends upon DNA replication.

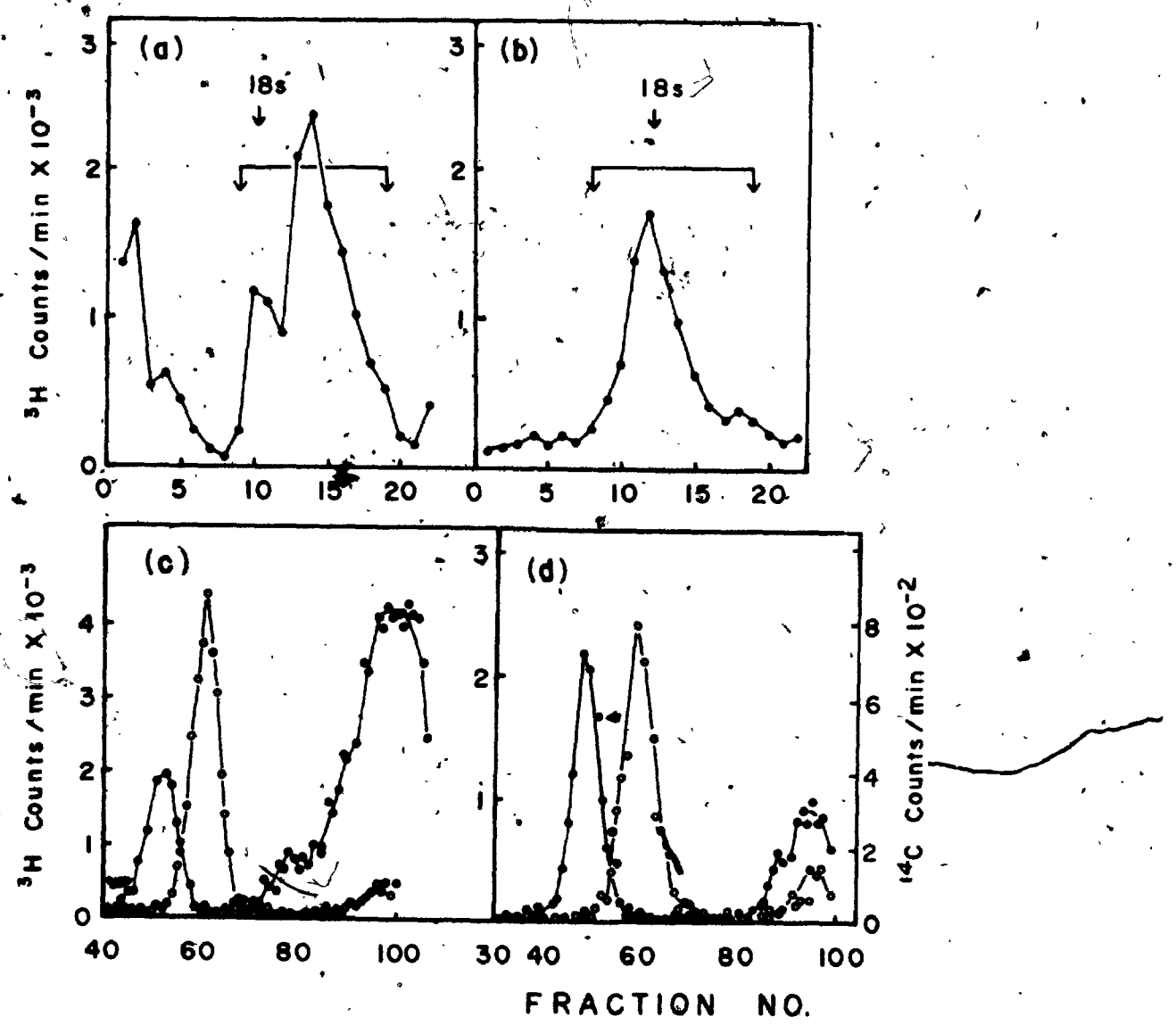
4. The replicative intermediate of form Ic DNA. Having established that the formation of component Ic occurs by a process dependent upon DNA replication, two alternative possibilities remained to explain the actual involvement of replication. I had shown in Part I of this section that the amount of closed-circular viral DNA formed in the absence of protein synthesis is limited only by the rate of initiation of new rounds of genome replication. Thus, once the synthesis of form I DNA is initiated, replication proceeds normally, but some protein synthesis-dependent process in the closure of daughter DNA may result in reduction of superhelicity of the progeny molecules. On the other hand, it was also considered possible that the normal completion of a round of replication in the absence of protein synthesis would yield progeny DNA of normal superhelix density. Superhelical turns could then be removed from these molecules by a process independent of replication. To differentiate between these alternatives, I examined the possibility that form I DNA is synthesized as an intermediate in the form I to Ic conversion.

Infected cells actively synthesizing viral DNA were treated for 90 minutes with cycloheximide and then

pulse-labeled for 4 minutes with [^{3}H] TdR. Velocity sedimentation at neutral pH revealed labeled material with the sedimentation properties of viral replicative intermediate DNA (RI) (Cheevers, Kowalski & Yu, 1972) and form Ic (Fig. 16(a)). CsCl-PDI analysis of this material (Fig. 16(c)) confirmed that a small amount of form Ic was generated during the pulse, but most of the DNA exhibited banding properties typical of viral RI (Roman, Champoux & Dulbecco, 1974). No detectable form I DNA was synthesized. In addition, viral RI chased in the absence of protein synthesis was entirely converted into closed-circular DNA of the form Ic conformation (Fig. 16(b) and (d)). Several experiments of the type shown in Fig. 16 have been done, varying the duration of the pulse label and the chase periods, and in no case has any form I DNA been detected (Cheevers, unpublished results). These results indicate that progeny form I DNA is not involved as an intermediate in the formation of component Ic.

This evidence supports the hypothesis that form Ic arises via an alteration of the closure of newly-replicated DNA rather than by removal of superhelical turns by a mechanism independent of replication. To verify this view, advantage was taken of the fact that the transition of form I DNA synthesis to form Ic in cycloheximide-treated cells occurs with first order exponential kinetics (See Fig. 25 & 26 of this section), which allows a choice

Fig. 16. Metabolic fate of viral RI in the absence of protein synthesis. Infected cells were pre-treated for 90 minutes with cycloheximide at 28 hours post-infection and pulse-labeled with [^{3}H] TdR (170 μCi/ml) for 4 minutes. The radioactive medium was then replaced with medium containing 2 x 10^{-5}M unlabeled TdR and cycloheximide. Viral DNA was isolated before (a) and after (b) the chase period by velocity sedimentation in neutral sucrose gradients. The indicated fractions were combined, and the DNA was concentrated by ethanol precipitation and analyzed by isopycnic centrifugation in CsCl-PDI as described in Fig. 11: (c) DNA from (a). (d) DNA from (b). (● - ●) [^{3}H] radioactivity. (O - O) [^{14}C] form I marker DNA.


(a)
18s
(b)
18s
(c)
(d)
^{3}H Counts / min X 10^{-3}
^{3}H Counts / min X 10^{-3}
^{14}C Counts / min X 10^{-2}
FRACTION NO.

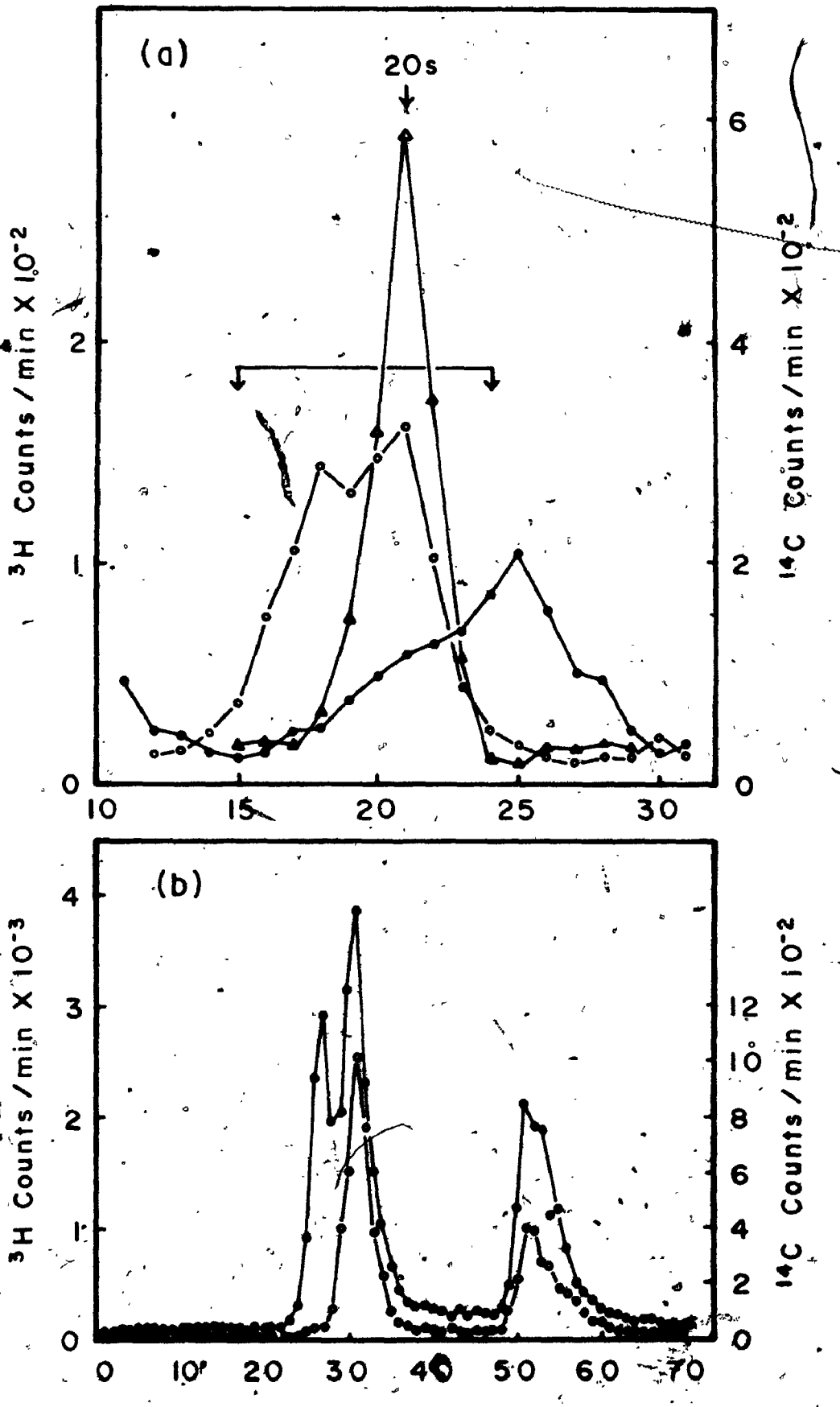
of conditions of treatment with cycloheximide in which both I and Ic DNA would be formed in predictable proportions. Thus the question was asked whether viral RI could be converted into a mixture of I and Ic, the proprotions of which would remain stable during a long chase in the continued absence of protein synthesis.

Cells were treated with cycloheximide under conditions of infection in which ~~form~~ Ic would be expected to represent about one-half of the closed-circular DNA being synthesized.. The cultures were pulse-labeled for 3 minutes with [^{3}H] TdR, and the newly-synthesized DNA was then followed for an additional 2-hour chase in the absence of protein synthesis.

Figure 17(a) shows that the sedimentation distribution of the pulse-labeled DNA was that of viral RI. During the chase in the presence of cycloheximide, this DNA was converted into material with the sedimentation properties of a mixture of forms I and Ic closed-circular species. Figure 17(b) confirms by CsCl-PDI analysis that the closed-circular viral DNA present after the chase was comprised of about 40% form Ic and 60% form I.

This experiment eliminates the possibility that superhelical turns may be removed from progeny DNA by a process independent of DNA replication. This is evident because the form I progeny DNA which resulted from

Fig. 17. Metabolic stability of form I DNA in the absence of protein synthesis. (a) Velocity sedimentation analyses of viral DNA. Infected cells were pretreated for 30 minutes with cycloheximide and pulse-labeled with [^{3}H] TdR (150 μCi/ml) for 3 minutes. Cultures were then harvested (● - ●) or chased for an additional 2 hours with medium containing unlabeled TdR and cycloheximide (o - o). (Δ-Δ) [^{14}C] form I marker DNA. (b) DNA isolated from cultures after the chase period (indicated in (a)) was analyzed by CsCl-PDI centrifugation as described in Fig. 10. (● - ●) [^{3}H] DNA. (o - o) [^{14}C] form I marker DNA.

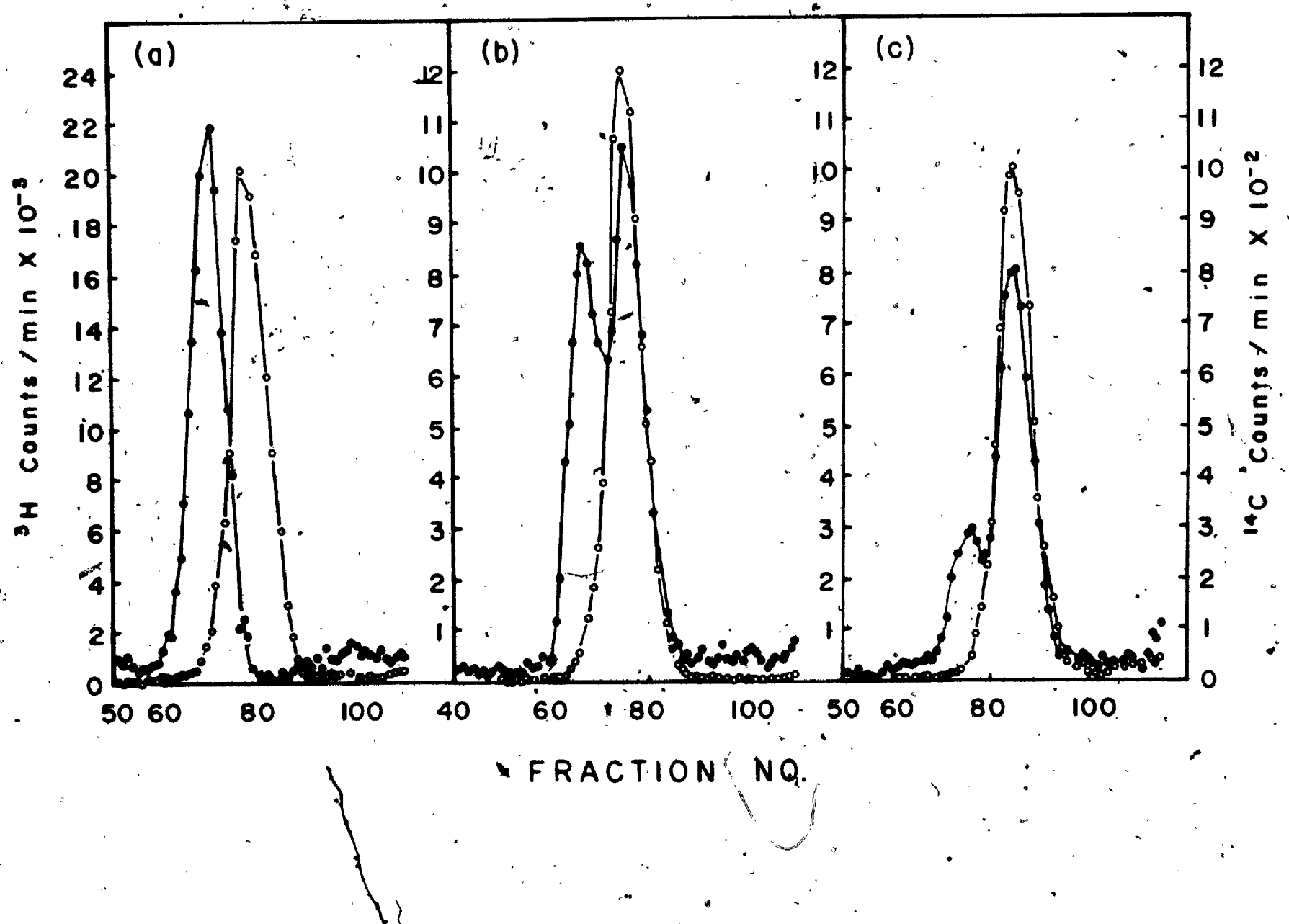

(a)
20s
^{3}H Counts / min X 10^{-2}
^{14}C Counts / min X 10^{-2}
0
1
2
0
2
4
6
10
15
20
25
30
(b)
^{3}H Counts / min X 10^{-3}
^{14}C Counts / min X 10^{-2}
0
1
2
3
4
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70

the completion of viral RI labeled during the 3-minute pulse was not converted to form Ic during the additional 2 hours in the presence of cycloheximide. Thus, we conclude that protein synthesis is required for the normal closure of newly-replicated polyoma DNA. After inhibition of protein synthesis by cycloheximide, some aspect of the closure mechanism is altered, resulting in the formation of progeny molecules exhibiting a marked deficiency in superhelicity.

5. Metabolic fate of form Ic DNA. The fate of form Ic DNA was examined in pulse-chase experiments in which protein synthesis was allowed to resume during the chase period. Infected cells were pretreated with cycloheximide and incubated with [^{3}H] TdR in the presence of cycloheximide to label newly-synthesized Ic. The cultures were then washed with warm medium without cycloheximide, and incubation was continued with this medium for up to 4 hours. Under these conditions, both protein synthesis and DNA synthesis returned to normal rates within less than 5 minutes after the removal of cycloheximide.

Figure 18 shows the distribution of labeled viral DNA in CsCl-PDI before and after chasing in the absence of cycloheximide. As expected, all of the DNA synthesized in the presence of cycloheximide banded in the position of form Ic (Fig. 18(a)). After a 2-hour

Fig. 18. Metabolic fate of form Ic DNA after removal of cycloheximide. Infected cells were pretreated with cycloheximide for 60 minutes and then labeled with [^{3}H] TdR (40 µCi/ml) between 30 1/2 and 31 1/2 hours post-infection in the continued presence of cycloheximide. Cultures were then harvested or chased with medium containing unlabeled TdR without cycloheximide for 1, 2, 3 or 4 hours. Viral DNA was isolated as described in Fig. 10 and analyzed by CsCl-PDI centrifugation as described in Fig. 11. (a) 1-hour pulse with [^{3}H] TdR in cycloheximide-treated cells. (b) 1-hour pulse with [^{3}H] TdR in the presence of cycloheximide followed by 2-hour chase in the absence of cycloheximide. (c) 1-hour pulse with [^{3}H] TdR in the presence of cycloheximide followed by 4-hour chase in the absence of cycloheximide. (● - ●) [^{3}H] DNA. (○ - ○) [^{14}C] form I DNA marker.

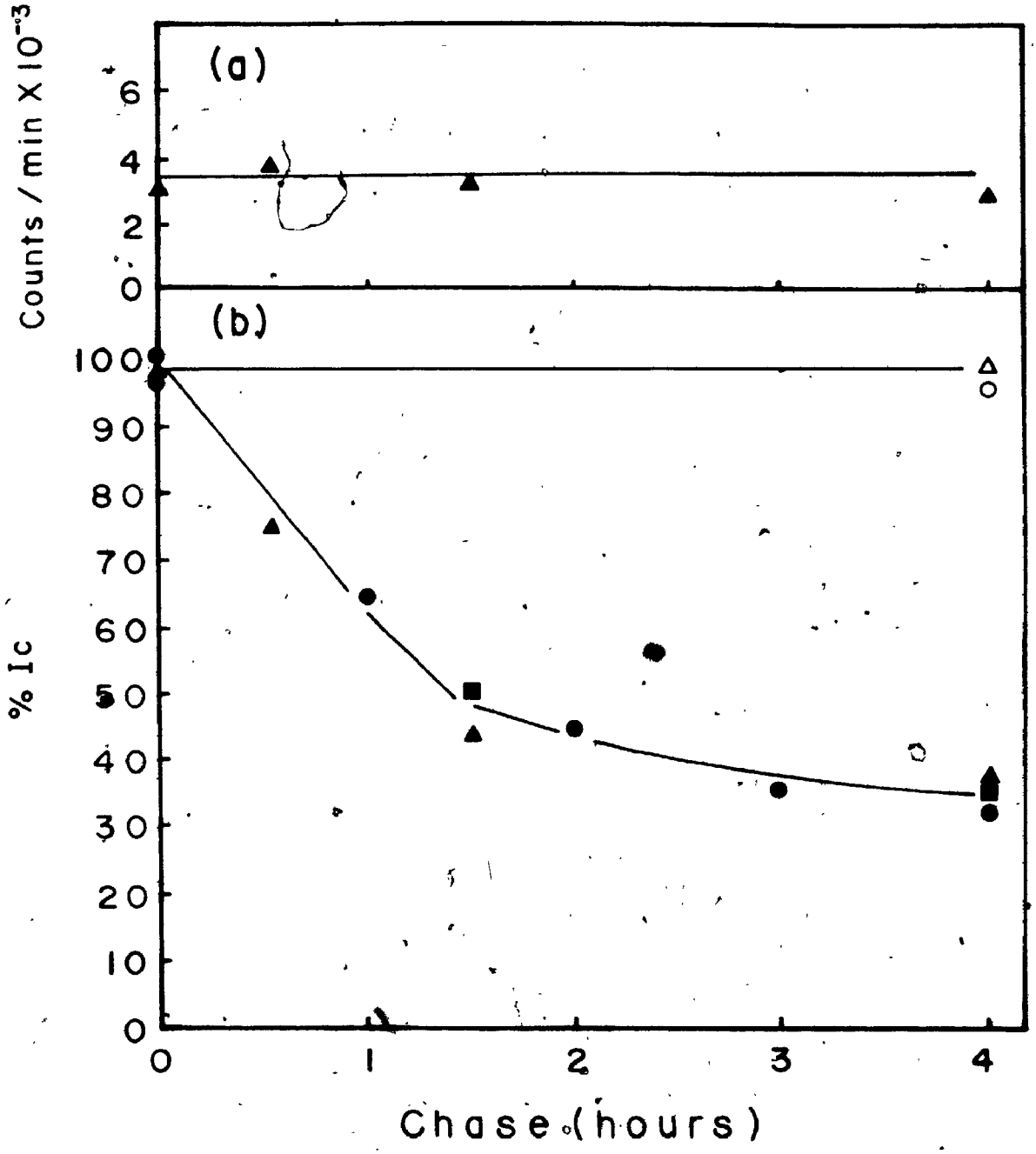

(a)
(b)
(c)
^{3}H Counts / min X 10^{-3}
^{14}C Counts / min X 10^{-2}
FRACTION NO.
0
2
4
6
8
10
12
14
16
18
20
22
24
1
3
5
7
9
11
40
50
60
80
100

chase, upon restoration of protein and DNA synthesis, both form I and Ic were apparent, form Ic accounting for almost half of the DNA (Fig. 18(b)). After a 4-hour chase (Fig. 18(c)), form Ic was reduced to one-third of the DNA, the remainder having been converted to form I.

Figure 19(b) shows the kinetics of re-acquisition by form Ic of the form I conformation upon reversal cycloheximide inhibition. About half of the form Ic was converted to form I at an approximately linear rate within 90 minutes. Thereafter, conversion was markedly slower; by 4 hours, 30-40% of the DNA still remained in the form Ic conformation.

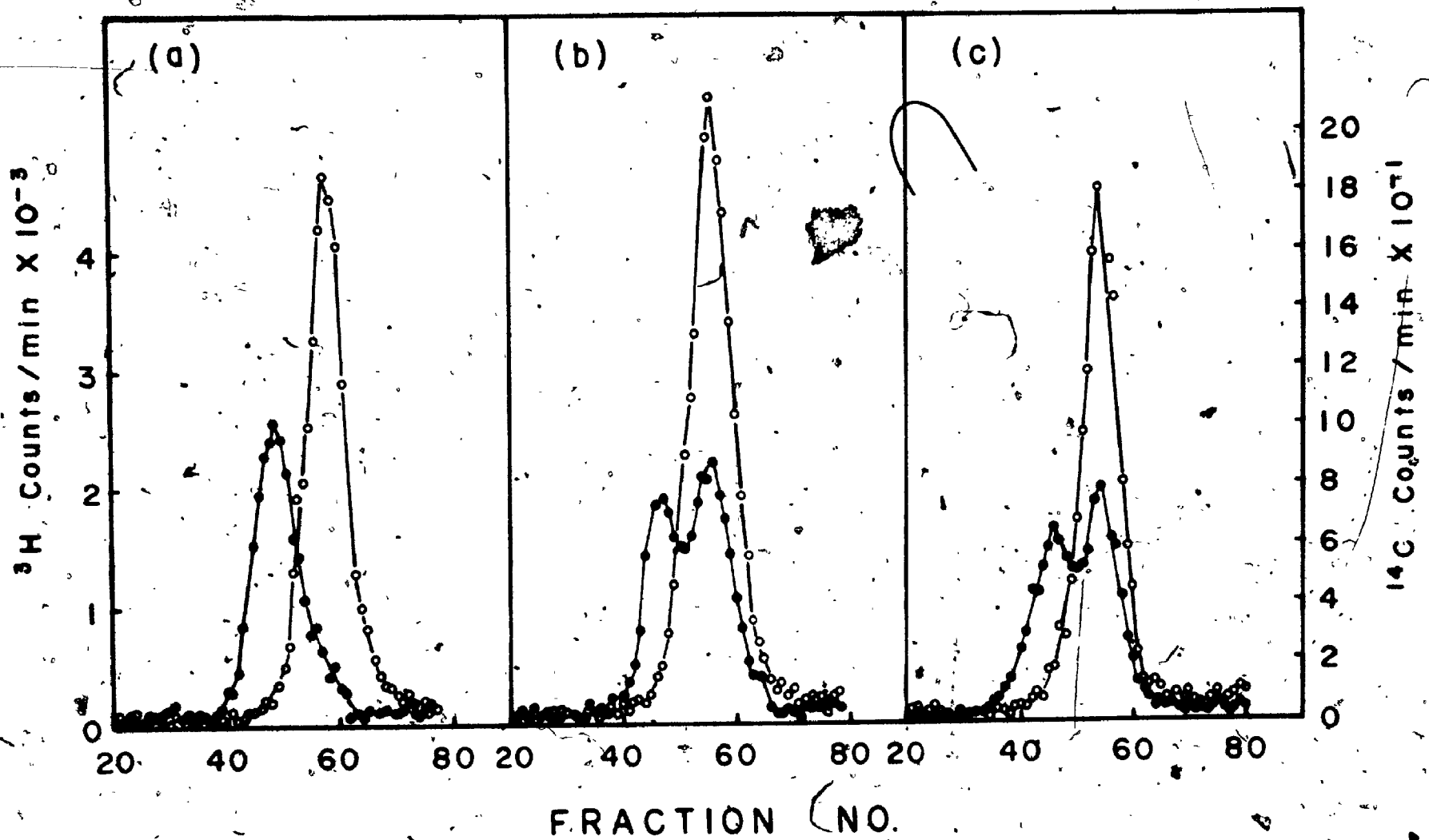
The involvement of replication in the Ic to I transition was examined by experiments in which form Ic was made in the presence of cycloheximide, and the labeled DNA chased under conditions of restored protein synthesis in the presence or absence of hydroxyurea (HU) or ara-C to inhibit DNA synthesis. Figure 20 shows the CsCl-PDI density gradients obtained from a typical experiment: 60% of form Ic made in the absence of protein synthesis (Fig. 20(a)) was converted into form I DNA during a 4-hour chase after reversal of cycloheximide inhibition (Fig. 20(b)). The presence of HU during the chase period had no effect on the proportions of I and Ic formed (Fig. 20(c)). Similar results were obtained using ara-C to inhibit DNA synthesis.

Fig. 19. Kinetics of formation of form I DNA from prelabeled form Ic upon restoration of protein synthesis. (a) Inhibition of [^{3}H] TdR incorporation into DNA by addition of excess unlabeled TdR (2×10^{-5}M). (b) Kinetics of reformation of component I DNA after removal of cycloheximide. Data were derived from three separate experiments as described in Fig. 18. (o - o,Δ - Δ) form Ic DNA chased in the presence of cycloheximide. (● - ●,▲ - ▲,■ - ■) form Ic DNA chased in the absence of cycloheximide.


(a)
(b)
Counts / min X 10⁻³
% Ic
Chase (hours)
0
2
4
6
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4

96

Fig. 20. Relationship between DNA replication and the reformation of component I DNA from prelabeled form Ic upon restoration of protein synthesis. Infected cultures were pretreated for 60 minutes with cycloheximide and labeled with [^{3}H] TdR (90 µCi/ml) at 26 1/2 - 28 1/2 hours post-infection. Cultures were either harvested (a) or chased for 4 hours with medium without cycloheximide in the absence (b) or presence (c) of 0.01 M HU. Viral DNA was isolated by sedimentation in neutral sucrose gradients and analysed by CsCl-PDI centrifugation as described in Fig. 1a. (● - ●) [^{3}H] DNA. (O - O) [^{14}C] form I DNA marker.


(a)
(b)
(c)
^{3}H Counts/min X 10^{-3}
^{14}C Counts/min X 10^{-1}
0
1
2
3
4
0
2
4
6
8
10
12
14
16
18
20
20
40
60
80
FRACTION NO.

These results show that superhelical turns may be introduced into form Ic DNA upon restoration of protein synthesis by some process independent of DNA replication. A subpopulation of Ic molecules, accounting for about one-third of the total, do not respond to cycloheximide reversal by re-acquisition of the normal superhelix density of polyoma DNA. These molecules are apparently stable end products of replication in the absence of protein synthesis.

Part III

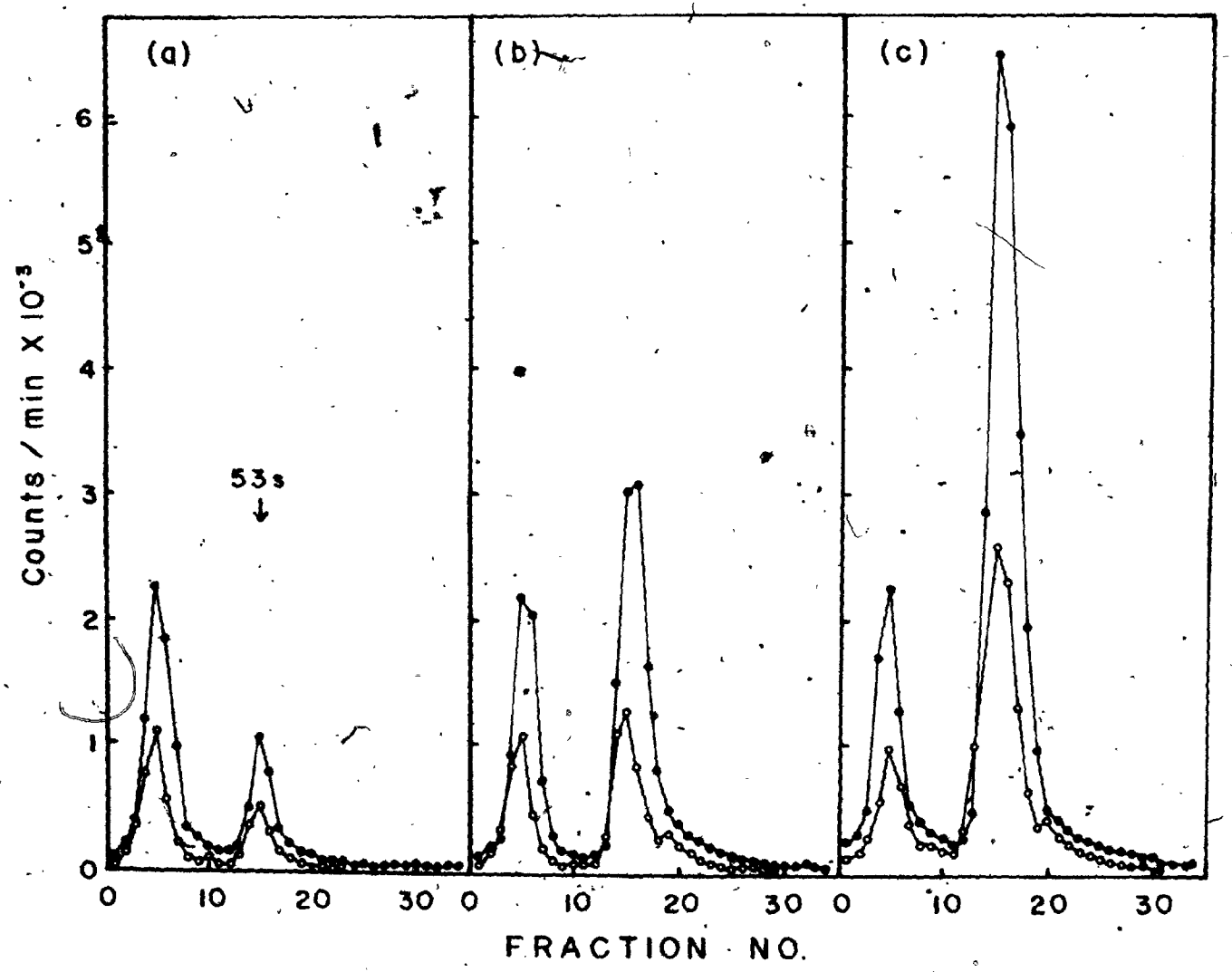
Two different requirements for protein synthesis during polyoma. DNA replication —— kinetic evidence.

1. Kinetics of inhibition of protein and viral DNA synthesis by cycloheximide. The experiment described in Fig. 22(a) shows that cycloheximide, at a concentration of 10 µg/ml, rapidly blocks the cumulative synthesis of protein in polyoma-infected cells. The rate of incorporation of [^{3}H] amino acids was reduced by 94% within 1 minute after the addition of cycloheximide and by 99% within 4 minutes (Fig. 23).

To measure the inhibition of viral DNA synthesis, medium containing [^{3}H] thymidine (TdR) with or without cycloheximide was added to infected cultures at 24 hours post-infection. At various times thereafter, treated and untreated cells were harvested, and labeled viral DNA was separated from high-molecular-weight cellular DNA by sedimentation in neutral sucrose gradients as previously described (Cheevers, Kowalski & Yu, 1972). The viral DNA was then isolated from appropriate neutral gradient fractions, and closed-circular components were separated by alkaline velocity sedimentation analysis. Under these conditions, both form I and form Ic are resistant to alkali-induced strand separation, and sediment at 53S (Bourgaux & Bourgaux-Ramoisy, 1972a, Cheevers, 1973).

Fig. 21 shows representative alkaline sedimentation profiles. The main point to be made is that

Fig. 21. Alkaline sedimentation analysis of viral DNA synthesized in untreated and cycloheximide-treated polyoma infected cells. Medium containing [^{3}H] TdR supplemented with unlabeled TdR with or without cycloheximide (final concentrations of 25 μCi/ml, 0.1 μg/ml and 10 μg/ml respectively) was added to infected cultures at 24 hours post-infection. At various times thereafter, viral DNA was isolated by neutral sucrose gradient sedimentation, denatured with NaOH and centrifuged in alkaline sucrose gradients as described in Materials and Methods. (a) 15 minutes. (b) 30 minutes. (c) 60 minutes. (• - •) [^{3}H] DNA, untreated cells. (o - o) [^{3}H] DNA, cycloheximide-treated cells.


(a)
(b)
(c)
53s
Counts / min X 10⁻³
0
1
2
3
4
5
6
0
10
20
30
FRACTION NO.

at all three time intervals (15 minutes, Fig. 21(a); 30 minutes, Fig. 21(b); 60 minutes, Fig. 21(c)), less radioactively-labeled 53S DNA was made in cycloheximide-treated cells than in untreated cells. Fig. 22(b) shows the cumulative synthesis of closed-circular viral DNA as determined by the methods described in Fig. 21. As expected, the incorporation of [^{3}H] TdR into viral DNA between 24 and 26 hours post-infection increased cumulatively at a progressively more rapid rate in untreated cells. Labeled DNA accumulated much more slowly in cycloheximide-treated cells.

In comparison to untreated controls, the rate of accumulation of viral DNA in the presence of cycloheximide was progressively reduced with time. This is more evident by the data of Fig. 23, which illustrates the slope of cumulative incorporation curves in treated cells as a function of that in untreated cells. From this calculation, it is obvious that viral DNA synthesis was inhibited by cycloheximide with complex kinetics. The rate of formation of 53S DNA was reduced by 40-50% within 15 minutes after the addition of cycloheximide. Thereafter, the rate of DNA synthesis declined more slowly, reaching a level of 10% of that in untreated cells only after about 2 hours.

It was shown in Part I of this section that polyoma DNA synthesis is inhibited by cycloheximide exclusively at the level of initiation of new rounds of

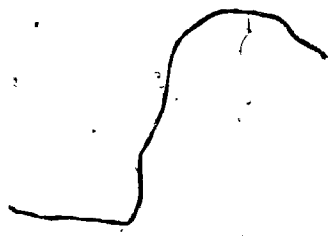


Fig. 22. Effect of cycloheximide on the cumulative synthesis of protein and closed-circular viral DNA in polyoma-infected cells. (a) Protein synthesis: Infected cultures were labeled at 24 hours post-infection with [^{3}H] amino acids (4 μCi/ml) with or without cycloheximide. Incorporation into the hot trichloroacetic acid-insoluble fraction was followed for 8 minutes. (● - ●) Untreated cultures. (o - o) Cycloheximide-treated cultures. (b) DNA synthesis: Infected cultures were labeled at 24 hours post-infection with medium containing [^{3}H] TdR (25 μCi/ml) and 0.1 μg/ml unlabeled TdR with or without cycloheximide. Incorporation into closed-circular viral DNA was measured after the indicated times by the procedures described in Fig. 1. (● - ●) Untreated cultures. (o - o) Cycloheximide-treated cultures.

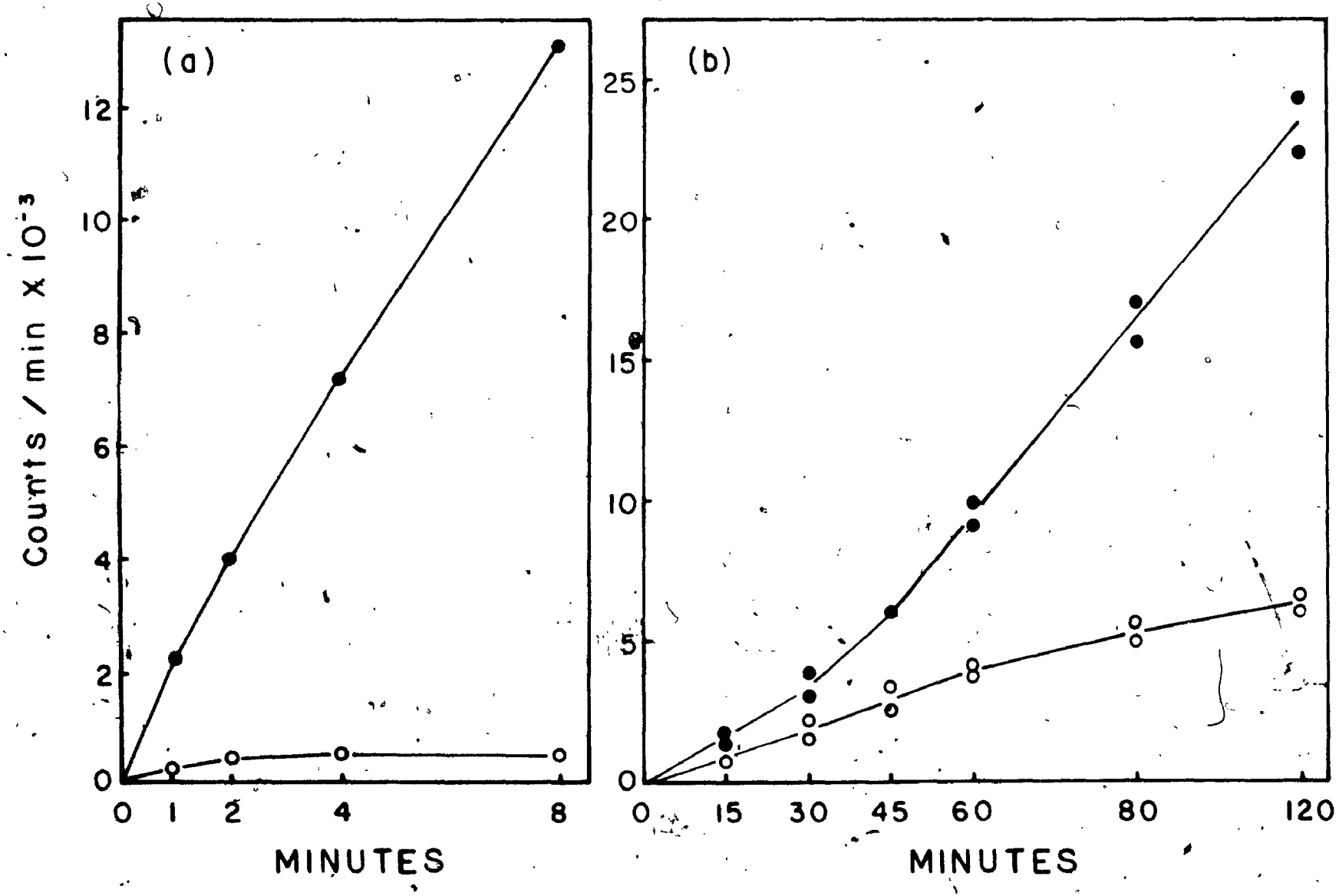
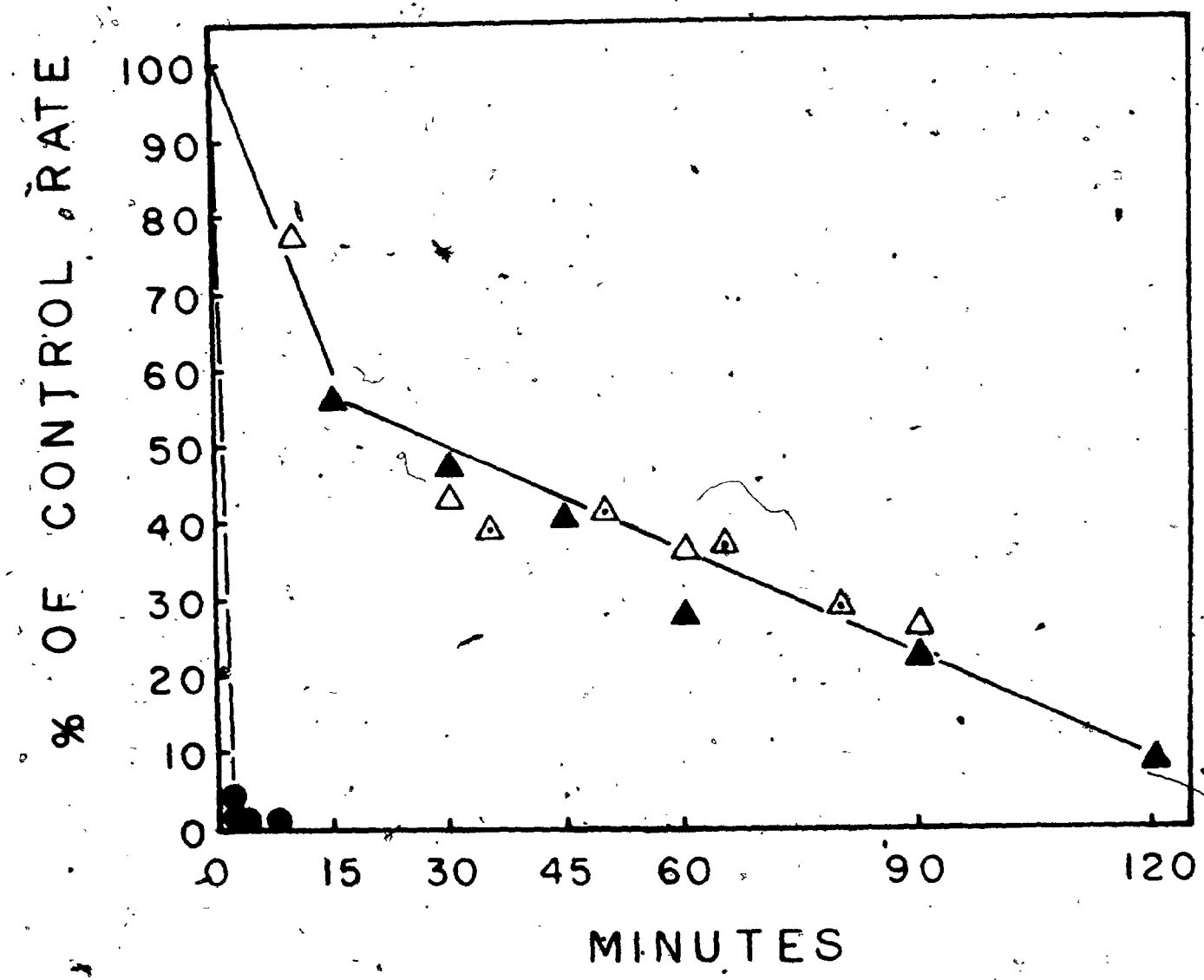

(a)
(b)
Counts / min X 10⁻³
0
2
4
6
8
10
12
0 1 2 4 8
MINUTES
0
5
10
15
20
25
0 15 30 45 60 80 120
MINUTES

Fig. 23. Kinetics of inhibition of protein and polyoma DNA synthesis by cycloheximide. The slope of cumulative incorporation curves in cycloheximide-treated cells for [^{3}H] amino acids (Fig. 22(a)) (● - ●) and [^{3}H] TdR (Fig. 22(b)) (▲ - ▲) are expressed as a function of the slope of incorporation curves in untreated cells. (Δ - Δ, ▲ - ▲) Additional experiments on viral DNA synthesis. ([^{3}H] TdR incorporation curves not shown).


% OF CONTROL RATE
100
90
80
70
60
50
40
30
20
10
0
0
15
30
45
60
90
120
MINUTES

genome replication. Thus, it appears that the initiation step in viral DNA replication requires the concurrent formation of protein(s). The results described here suggest the involvement of a protein requirement for initiation of viral DNA replication since this process cannot be maintained at a normal rate for more than a few minutes in the absence of protein synthesis.

2. Synthesis of form Ic viral DNA in cycloheximide-treated cells. In the absence of protein synthesis, newly-synthesized form I polyoma DNA is replaced by a viral DNA component form Ic. This DNA is a monomeric closed-circular DNA species characterized by a two-thirds reduction in superhelicity as compared to form I (See Part II of this section). It is synthesized in the presence of cycloheximide on pre-existing form I templates without the intervention of progeny form I DNA as an intermediate; thus it arises by alteration of a protein synthesis dependent step in the closure of the terminal intermediate of viral DNA replication. In order to measure the rate at which the synthesis of viral DNA is diverted from the formation of component I to component Ic, infected cells were pretreated for various periods of time with cycloheximide and incubated with [^{3}H] TdR to label replicating viral DNA. The viral DNA was separated from high-molecular-weight cellular DNA by sedimentation in neutral sucrose gradients and then resolved into components I and Ic by isopycnic centrifugation in CsCl-

PDI gradients (Cheevers, 1973; Yu, Kowalski & Cheevers, 1975 a). Results are shown in Fig. 24. As expected, all of the closed-circular viral DNA synthesized in the absence of cycloheximide exhibited the form I conformation (Fig. 24(a)). With increasing time of pretreatment with cycloheximide, however, the proportion of labeled DNA with the superhelix density of form I progressively decreased; concomitantly, form Ic DNA, with a lower superhelix density, increased (Fig. 24(b), (c) and (d).

The rate at which form I DNA was replaced by Ic in this experiment is shown in Fig. 25, expressed as a semi-logarithmic plot of the percentage of form I remaining after each pretreatment time. For comparison, the inhibition of [^{3}H] TdR incorporation into total closed-circular DNA, measured by alkaline sedimentation and CsCl-PDI centrifugation, is also shown. It is clear from these results that in the absence of protein synthesis viral DNA replication is diverted from the formation of component I to component Ic with first-order exponential kinetics, whereas the rate of initiation of viral DNA synthesis is inhibited much faster and with different kinetics (see also Fig. 23).

From Fig. 25 it may be estimated that about 30 minutes in the presence of cycloheximide are required for one-half of newly-synthesized DNA to acquire the superhelix density of form Ic. If this is correct, extrapolation of the decay curve predicts that form I DNA synthesis should

Fig. 24. Isopycnic centrifugation in CsCl-PDI gradients of polyoma DNA synthesized in cycloheximide-treated cells. Infected cells were incubated with medium or medium containing cycloheximide for various times labeled with [^{3}H] TdR (25 μCi/ml) at 27-28 hours post-infection. Viral DNA was isolated by sedimentation in neutral sucrose gradients, and the buoyant density distribution of closed-circular components was then determined by isopycnic centrifugation in CsCl-PDI gradients (rotor SW50.1) (see Vinograd, Lebowitz & Watson, 1968; Champoux & Dulbecco, 1972) (a) Untreated cells. (b) 10-minute pretreatment with cycloheximide. (c) 30-minute pretreatment with cycloheximide. (d) 60-minute pretreatment with cycloheximide. (● - ●) [^{3}H] DNA. (o - o) [^{14}C] form I viral DNA marker, isolated by neutral sedimentation analysis of DNA extracted from infected cells labeled with [^{14}C] TdR from 10-30 hours post-infection (see Vinograd, Lebowitz & Watson, 1968).

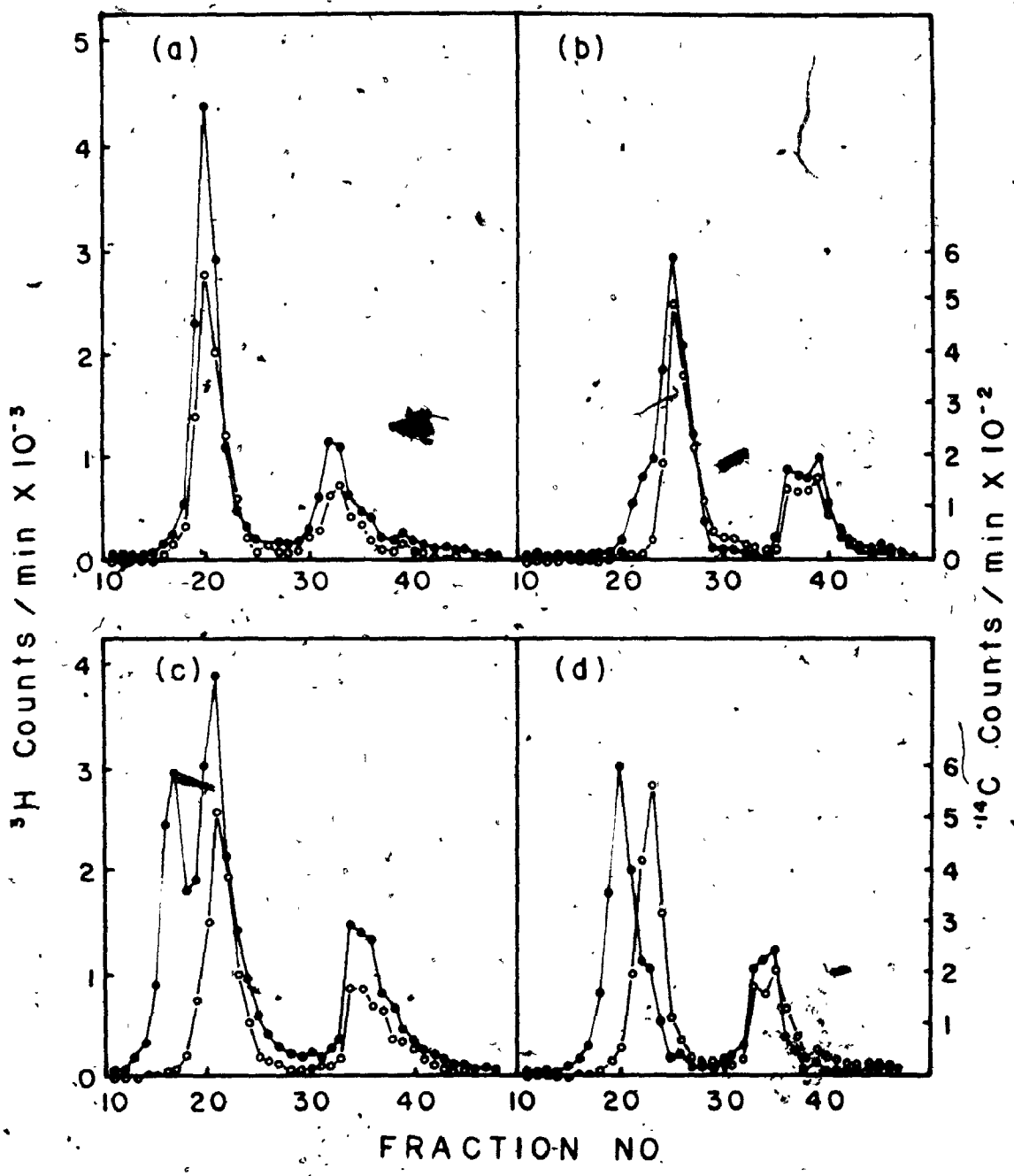
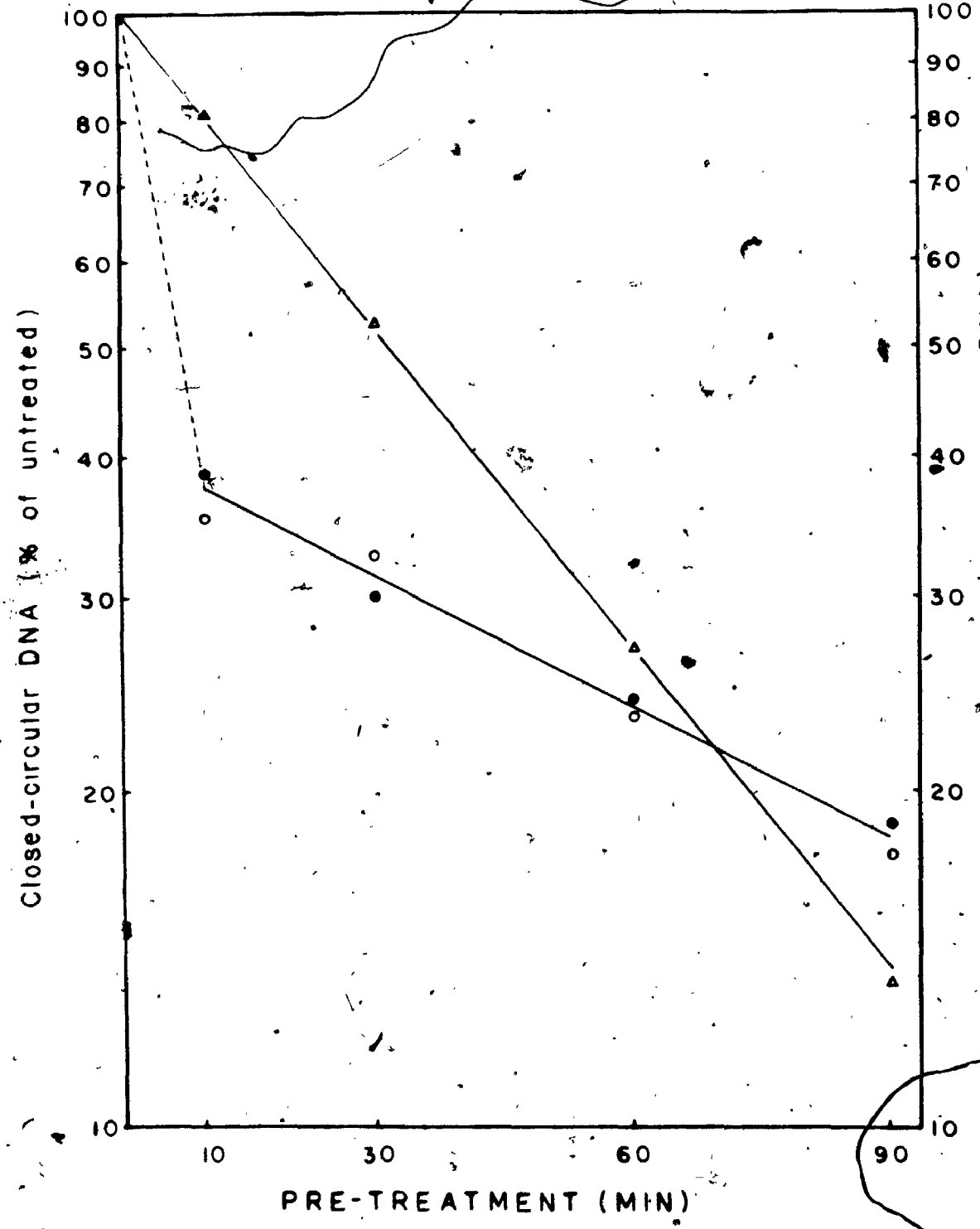

(a)
(b)
(c)
(d)
^3H Counts / min X 10^{-3}
^{14}C Counts / min X 10^{-2}
FRACTION NO
10
20
30
40

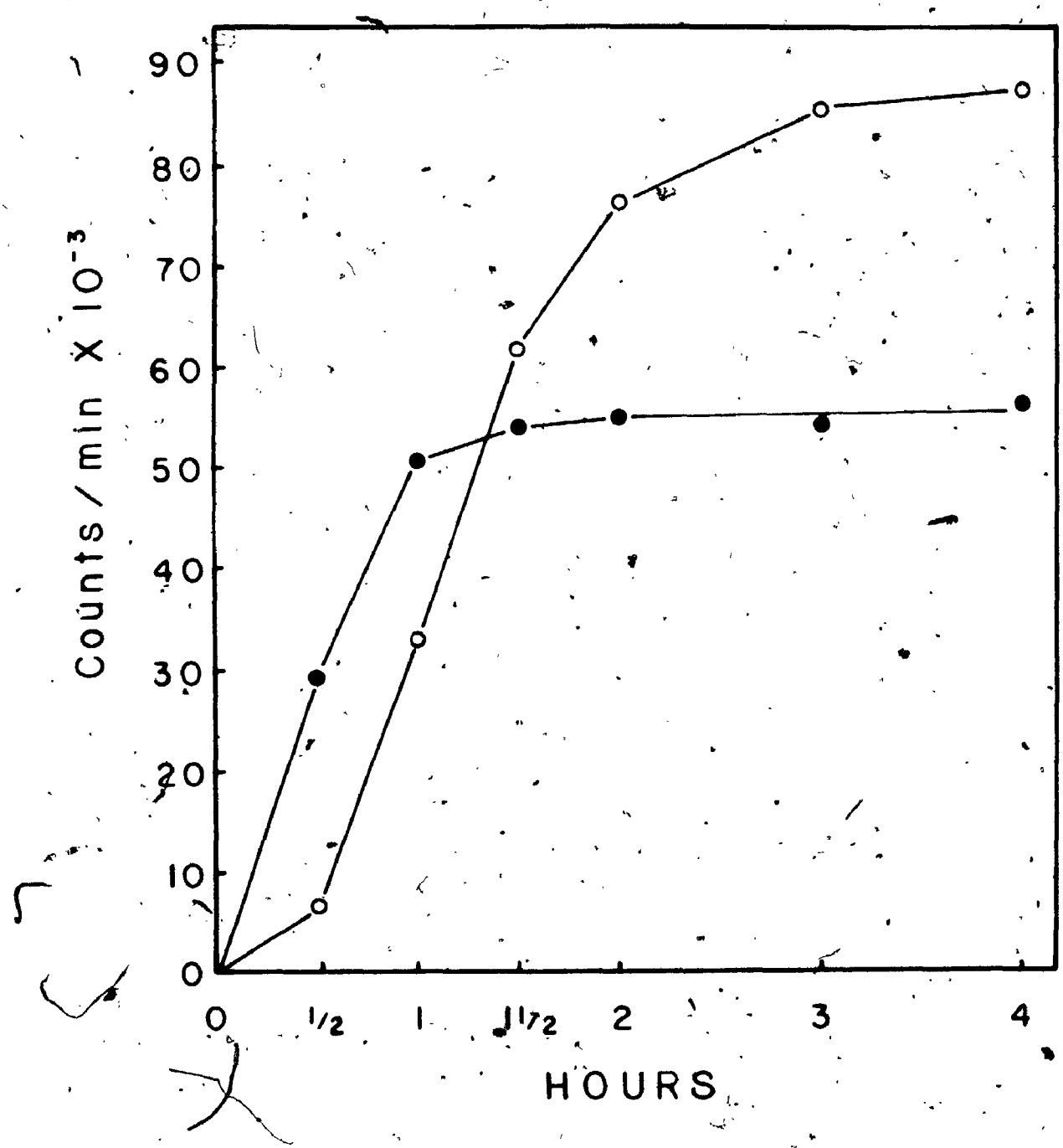
Fig. 25. Rate of inhibition of form I DNA synthesis by cycloheximide. Infected cells were pretreated for various times with cycloheximide and labeled with [^{3}H] TdR as described in Fig. 24. Viral DNA isolated by sedimentation in neutral sucrose gradients was concentrated by ethanol precipitation and dissolved in 0.3M NaCl - 0.001M EDTA - 0.01M Tris, pH 8.1. An aliquot of each sample was denatured with NaOH, and total 53S closed-circular DNA was separated by velocity sedimentation in alkaline sucrose gradients (● - ●). The remainder of each sample was analyzed by isopycnic centrifugation in CsCl-PDI to distinguish form I and form Ic components (buoyant density distributions for pretreatment times of 0, 10, 30 and 60 minutes are shown in Fig. 24) (○ - ○) Determination of total closed-circular DNA from CsCl-PDI gradients. (Δ - Δ) Estimation of form I DNA from CsCl-PDI gradients.


100
90
80
70
60
50
40
30
20
10
Closed-circular DNA (% of untreated)
Form I (% of closed-circular DNA)
10
30
60
90
PRE-TREATMENT (MIN)

essentially stop after 90-120 minutes in the absence of protein synthesis. This was confirmed by the experiment described in Fig. 26, in which the cumulative synthesis of forms I and Ic was followed in the presence of cycloheximide. Both species of closed-circular viral DNA were synthesized for approximately the first 90 minutes in cycloheximide-treated cells. Thereafter, essentially all of the newly-synthesized viral DNA was of the form Ic conformation.

I conclude from these findings and those in Part II of this section that in the absence of protein synthesis, the synthesis of polyoma DNA is diverted from the formation of normal component I to component Ic according to the exponential decay of protein(s) involved in the closure of the terminal intermediate of viral DNA replication. The kinetics of formation of component Ic suggest that normal closure requires the synthesis of a single protein with an average lifetime of approximately 1 1/2 hours.

Fig. 26. Accumulation of form I and form Ic viral DNA in cycloheximide-treated cells. Medium containing [^{3}H] TdR (50 μCi/ml) supplemented with 0.1 μg/ml unlabeled TdR and cycloheximide was added to infected cultures at 26 hours post-infection. At the indicated times, closed-circular viral DNA was isolated and resolved into form I and Ic components by centrifugation in CsCl-PDI gradients. (• - •) form I DNA. (o - o) form Ic DNA.


Counts / min X 10^{-3}
90
80
70
60
50
40
30
20
10
0
0
1/2
1
1 1/2
2
3
4
HOURS

DISCUSSION

Polyoma virus DNA replication was shown to be inhibited by cycloheximide (Branton, Cheevers & Sheinin, 1970; Branton, 1972; Branton & Sheinin, 1973; Cheevers, 1973; Green, 1972; Kang et al., 1971). While Bourgaux and Bourgaux-Ramoisy (1972) demonstrated that monomeric viral DNA synthesized in polyoma virus-infected cells in which protein synthesis has been blocked possessed a deficiency in superhelicity. The proportion of oligomeric viral DNA is also increased in the absence of protein synthesis, and these molecules also exhibit a reduction in superhelical turns (Bourgaux, 1973; Cheevers, unpublished data). Similar effects have also been noted in SV40-infected cells for both monomers (White & Eason, 1973) and oligomers (Jaenisch & Levine, 1973). The purpose of the present work was twofold: to determine the mechanism of inhibition of viral DNA synthesis in cycloheximide-treated cells and to determine the mechanism of formation of closed-circular polyoma DNA made in the presence of cycloheximide which I termed component Ic. With respect to elucidating the mechanism of DNA synthesis inhibition by CX, three molecular parameters of the replication cycle of viral DNA were considered :

(i) initiation of each new round of DNA synthesis,

(ii) elongation of nascent DNA strands of replicative intermediates and (iii) maturation of newly-synthesized DNA into closed-circular progeny molecules.

Saturation labeling experiments indicate that in cycloheximide-treated cells the size of the replicating pool of polyoma DNA is reduced by the amount consistent with the inhibition of ^{3}H-TdR incorporation, whereas the rate of turnover of replicative intermediates is unaffected. Pulse-chase studies of the kinetics of formation of closed-circular DNA and of nascent DNA strand elongation within the population of replicating molecules show that these processes proceed at a normal rate in the presence of cycloheximide. Therefore, the reduction in the size of the replicating pool is interpreted as indicative of a cycloheximide-induced block in the initiation of viral DNA synthesis. From these results I conclude that protein synthesis is required only for the initiation of each new round of replication; the completion of viral DNA molecules already being replicated is independent of concurrent protein synthesis.

These findings may be interpreted to support the view that only one viral gene product is involved in the control of polyoma DNA replication. A viral-specific "initiator" protein, required to start each new round of viral DNA replication, has been inferred from analyses of temperature-sensitive polyoma mutants of the ts-a type

(Francke & Eckhart, 1973) and SV 40 complementation group A mutants (Tegtmeyer, 1972). Francke and Hunter (1974b) using an *in vitro* DNA-synthesizing system, have shown that synthesis of the DNA of polyoma ts-25 (a ts-a group mutant) is not deficient in any parameter of the maturation of RI. From this, they attributed an initiation function to the ts-25 protein. Thus, it is reasonable to suppose from the present results that cycloheximide blocks the synthesis of viral "initiator", since host proteins cannot initiate viral DNA replication (Tegtmeyer, 1972).

Previous work has indicated that the timing of the replication of some regions of host DNA in polyoma-infected cells depends directly upon initiation of viral DNA synthesis (Cheevers & Hiscock, 1973; Cheevers, Kowalski & Yu, 1972). In cycloheximide-treated cells, viral DNA synthesis is inhibited with "step-down" kinetics coordinately with cellular DNA being replicated late in the infection (Cheevers, unpublished data). Thus, viral and cellular DNA replication are apparently coupled by some mechanism involving the concurrent synthesis of protein.

In view of the above considerations, the present results suggest that the initiation of new rounds of polyoma DNA replication and the initiation of at least a portion of cellular DNA replicons are regulated by a common mechanism, the elements of which include the continuous formation of an "initiator" protein. The most

obvious model arising from this is that viral DNA replication is initiated from an integrated state. If this is indeed the case, it is possible that viral-specific "initiator" could control the initiation of cellular DNA regions adjacent to the site of integration of viral DNA. However, it is not known whether the replication of viral DNA depends upon its prior integration into host DNA. Further work using temperature-sensitive mutants of polyoma virus defective in the initiation of viral DNA synthesis can possibly resolve this particular question. Another problem concerns the role of histones in the coordinate regulation of viral and cellular DNA synthesis. Cheevers (unpublished data) and others (Butler & Mueller, 1973; Weintraub, 1972; Weintraub, 1973; Weintraub & Holtzer, 1972) have obtained suggestive evidence that these proteins are involved in the control of eukaryotic DNA replication. Further, it has recently been shown that polyoma DNA is replicated in close association with host chromatin in the form of nucleoprotein particles containing histones (Seebeck & Weil, 1974).

At present, data are insufficient to define the mechanism of coordinate control of viral and cellular DNA synthesis in polyoma infection. An important question is the mechanism of inhibition of cellular DNA synthesis by cycloheximide. Although histones are probably involved in the control of DNA chain elongation (Weintraub, 1973),

inhibition of the synthesis of this specific class of proteins may not account for the entire effect of cycloheximide on DNA replication. In fact, evidence has been published indicating that in at least one primitive eukaryote only the initiation of replicons is affected (Hereford & Hartwell, 1973), which, as I have shown in this thesis, is also the mechanism of inhibition of polyoma DNA synthesis by the same drug. This means of coupling of protein synthesis and DNA replication is well-documented for bacteria (Lark, 1969). In other cases for eukaryotes, protein synthesis is reportedly required for both initiation and completion of DNA replication (Cummins, 1969; Hereford & Hartwell, 1973; R. Hand, personal communication; Cheevers and Kowalski, unpublished data). Clearly, the involvement of protein synthesis in the control of DNA replication in polyoma-infected cells is a complex process.

Towards elucidating the mechanism of component Ic (closed-circular polyoma DNA monomers made in the presence of CX) formation, experiments in which newly-replicated form I DNA was chased in cycloheximide-treated cells indicate that form Ic arises from pre-existing form I molecules. Approximately one-third of the form I DNA replicated during a 2-hour interval is converted to form Ic during a subsequent 2-hour chase in the absence of protein synthesis. This proportion is not significantly changed by increasing the chase time. The point to be

made from this experiment is that only one-third of the form I molecules can be traced into the Ic population at a time when essentially 100% of viral DNA being replicated is accounted for by the formation of Ic.

The most obvious explanation of this finding assumes that form Ic arises by replication on form I templates, since the rate of initiation of new rounds of viral genome replication is inhibited by cycloheximide (see Results, part I) by an amount consistent with the proportion of form I DNA involved in the formation of Ic (see Figs. 12 & 13). The involvement of replication in the conversion of form I to Ic was confirmed by the sensitivity of this process to the inhibition of DNA synthesis by ara-C.

Two alternative mechanisms were considered to account for the synthesis of form Ic. It was assumed that form I DNA molecules whose synthesis had already been initiated at the time of addition of cycloheximide as well as those initiated after the inhibition of protein synthesis would finish replication normally (See Results, Part I). Thus, form Ic could arise either by some alteration of the closure of daughter molecules or by removal of superhelical turns from newly-synthesized form I DNA. The latter possibility seemed particularly interesting in view of the discovery by Wang (1971) of protein ω in *Escherichia coli*, which removes superhelical turns in closed-circular DNA without introducing permanent strand scissions. Similar "untwisting" activities have been found in extracts of

mouse (Champoux & Dulbecco, 1972) and human cells (Keller & Wendel, 1974), as well as in nucleoprotein complexes containing replicating SV40 DNA (Sen & Levine, 1974). Thus, it seemed plausible that cycloheximide may inhibit the formation of some protein in polyoma-infected cells which normally acts to prevent relaxation of newly-synthesized progeny DNA. However, this idea was not supported, as no evidence was found to suggest that superhelical turns are removed from newly-synthesized DNA by a mechanism independent of replication. In fact, the data indicate that forms I and Ic arise from a replicative intermediate population. Inhibition of protein synthesis gradually shifts the synthesis of viral DNA from form I to Ic (Fig. 26). Under conditions of treatment with cycloheximide in which viral RI is chased into a mixture of closed-circular progeny molecules, the proportions of forms I and Ic are stable in the continued absence of protein synthesis. Thus, we conclude that the formation of component Ic viral DNA results from an alteration of some protein synthesis-dependent process in the closure.

Upon restoration of protein synthesis, approximately two-thirds of form Ic molecules return to the form I conformation by a process independent of DNA synthesis. This result supports previous work with SV40 indicating that supercoiling of viral DNA does not depend upon replication (White & Eason, 1973). The failure of one-

third of the form Ic molecules to assume a normal superhelix density upon restoration of protein synthesis is not understood. This is not due, however, to incomplete reversal of the effect of cycloheximide. These molecules may be substituted with cellular DNA sequences, arising by cycloheximide-induced enhancement of excision of integrated viral DNA, since high multiplicity passage yields such DNA molecules which band at the position of form Ic in CsCl-PDI (Hiscott, unpublished data). This possibility is being further investigated by him in this laboratory using reassociation kinetics.

Figure 27 shows a diagrammatic summary of the results of this work, which assumes that form II is a terminal intermediate of polyoma DNA replication (Fareed, McKerlie & Salzman, 1973). Upon inhibition of protein synthesis, the synthesis of polyoma DNA is limited by a reduced rate of initiation of new rounds of genome replication (See Results, Part I; step 1). Form I DNA molecules initiated in the absence of protein synthesis finish replication normally, yielding closed-circular progeny DNA (See Results, Part I). Protein synthesis is also required to maintain the superhelix density of progeny DNA. In its absence (step 2) the synthesis of viral DNA shifts from the formation of normal form I progeny to form Ic, a monomeric closed-circular viral DNA exhibiting a marked reduction in superhelical turns. Form Ic is the product of improper closure of the terminal

3 3

OF/DE

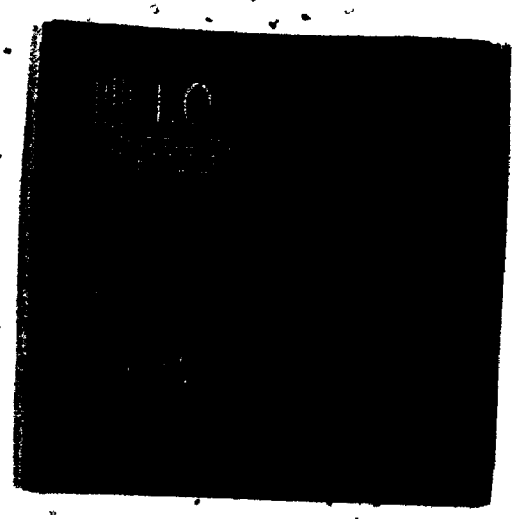
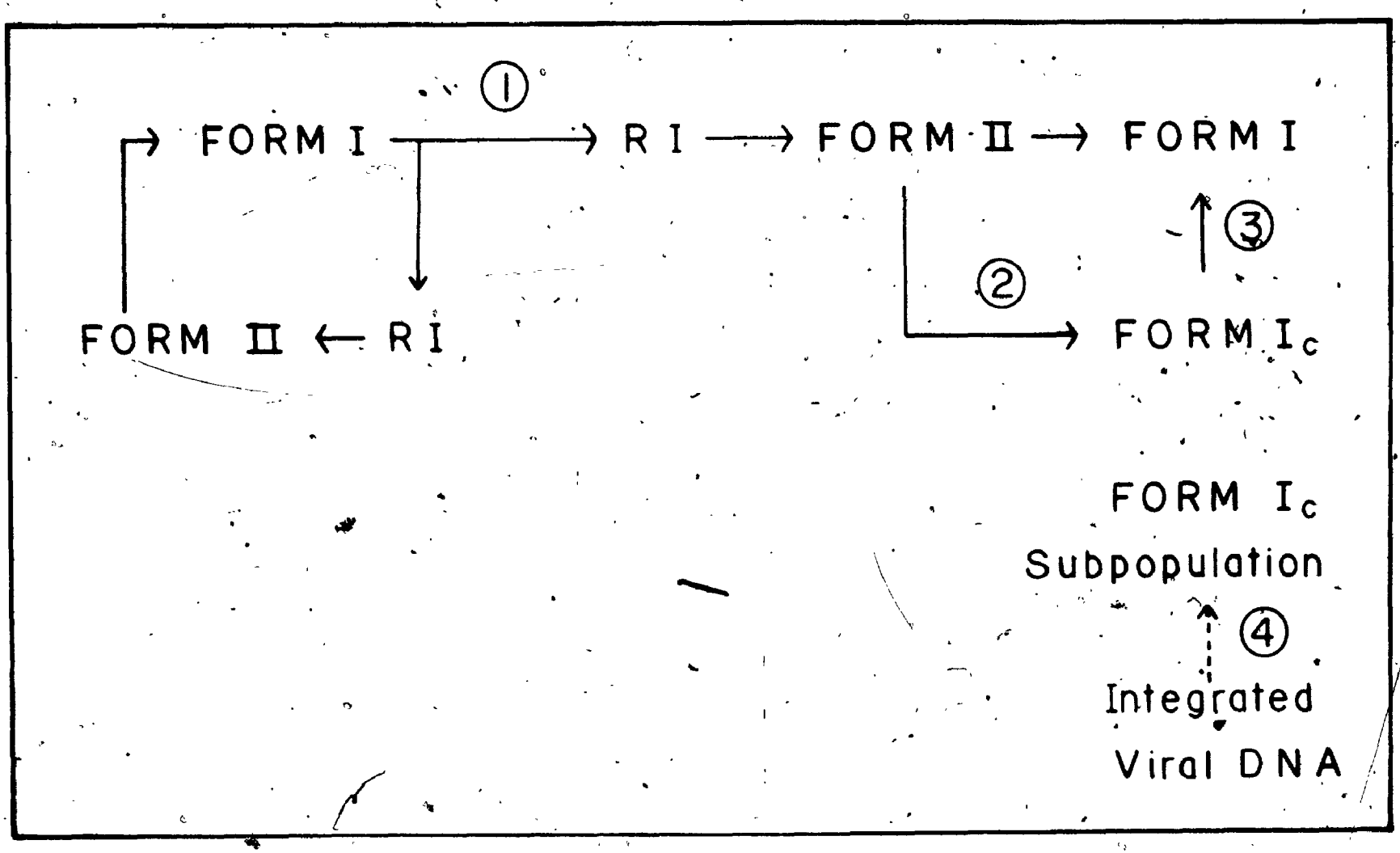


Fig. 27. Model for the replication of polyoma DNA in the absence of protein synthesis. The normal replication cycle of viral DNA generates form II terminal intermediates via elongation of closed-circular RI molecules. Form II is then closed and supercoiled to yield form I progeny DNA. In the absence of protein synthesis, the rate of initiation of new rounds of genome replication is inhibited (step 1), a process which governs the amount of DNA synthesized. In addition, residual viral DNA synthesis is diverted from the formation of component I to component Ic by alteration of the closure of the terminal intermediate. Form Ic, which is deficient in superhelical turns by about two-thirds comparison to form I, may recover the tertiary structure of form I upon restoration of protein synthesis (step 3). This process does not require DNA replication. A subpopulation of form Ic, which resists the re-introduction of superhelical turns, may arise by cycloheximide-induced enhancement of excision of integrated viral DNA sequences (step 4).


FORM I
①
RI
FORM II
FORM I
③
②
FORM II
RI
FORM Ic
FORM Ic
Subpopulation
④
Integrated
Viral DNA

intermediate of replication. Upon restoration of protein synthesis two-thirds of form Ic re-acquires a normal superhelix density by a process not requiring DNA synthesis (Step 3). The subpopulation of form Ic which does not return to the form I conformation may arise by cycloheximide-induced enhancement of the excision of integrated viral DNA sequences (step 4).

Thus, the present study has established that the control of autonomous polyoma DNA replication involves at least two requirements for concurrent protein synthesis: one which maintains the normal initiation rate for new rounds of genome replication (See Results, Part I), probably also coordinates the initiation of cellular DNA replicons late in the infection cycle (Cheevers, Kowalski & Yu, 1972). Protein synthesis required during the closure of newly-synthesized terminal intermediates of viral DNA is also involved in maintaining the superhelicity of progeny form I molecules. On the basis of the inhibition kinetics for these processes after interruption of protein synthesis by cycloheximide, I conclude that the formation of at least two distinct proteins is required. Essentially continuous protein synthesis is needed for the initiation of viral DNA replication since this process cannot be maintained at a normal rate for more than approximately 10 minutes in the presence of cycloheximide. Closure, on the other hand, is much more stable after treatment of

infected cells with this inhibitor. This process decays with first-order exponential kinetics, consistent with the involvement of a protein having an average lifetime of approximately 1 1/2 hours.

Within 15 minutes after the addition of cycloheximide, the rate of synthesis of closed-circular polyoma DNA is reduced by approximately one-half via inhibition of the initiation step in viral DNA replication. Following this initial rapid decay, viral DNA synthesis declines much more slowly, reaching a level equivalent to only 10% of the untreated cells after about 2 hours. This complex pattern of inhibition is not understood. Although it is probable that rounds of viral genome replication can continue at a lower rate for some time after the inhibition of new protein synthesis, as postulated by Branton (1972). Weintraub and Holtzer (1972) have obtained qualitatively similar results in eukaryotic cells treated with cycloheximide or puromycin. These authors termed this type of inhibition pattern of DNA synthesis "step-down". Similarly, Stanners and Thompson (1974) have shown that DNA synthesis is inhibited with "step-down" kinetics after interruption of protein synthesis in cells temperature-sensitive for leucyl-transfer RNA synthetase. Cheevers and Kowalski (unpublished data) have obtained the same results for cellular DNA synthesis in both normal and polyoma-infected exponential-phase mouse embryo cultures.

Weintraub and Holtzer (1972) attributed the

entire effect of the inhibition of protein synthesis on eukaryotic DNA replication to a reduction in the average rate of DNA strand elongation within replicons. This conclusion has been sustained in several other studies (Gautschi, 1974; Gautschi & Kern, 1973; Gautschi, Kern & Painter, 1973) but questioned by at least three groups (Hand & Tamm, 1973; Hereford & Hartwell, 1973; Hori & Lark, 1973). Mainprize and Cheevers (manuscript in preparation), show that cycloheximide does in fact reduce the rate of cellular DNA strand elongation, but this effect is secondary to an almost immediate block in the initiation of replicons. Thus, the primary effect of cycloheximide on both cellular and viral DNA replication is probably at the level of initiation.

From these and previous considerations on the temporal correlation of cellular and viral DNA synthesis in polyoma-infected cells (Cheevers, Kowalski & Yu, 1972; Cheevers & Hiscock, 1973), I propose that the initiation of new rounds of viral DNA replication and the initiation of at least a portion of cellular DNA replicons are regulated by a common control mechanism, the elements of which include the continuous formation of an initiator protein. The possible role of integration of viral DNA sequences into host DNA in this process could be investigated in the future using temperature-sensitive polyoma virus mutants defective in the initiation of viral DNA synthesis.

Involvement of proteins in the generation of the normal tertiary structure of form I polyoma DNA is not understood. It is possible that this protein functions as an inhibitor of other proteins which are known to remove superhelical turns from closed-circular DNA (Champoux & Dulbecco, 1972; Keller and Wendel, 1974). However, this mechanism cannot be simple competition between "untwisting" proteins and their inhibitors since the "untwisting" activity does not function to remove superhelical turns from newly-synthesized form I viral DNA in the absence of protein synthesis. A more likely possibility is that "untwisting" proteins are not involved in the generation of the superhelical structure of progeny DNA, but rather are enzymatic in nature and serve to create localllized regions of unwinding during DNA replication (Champoux & Dulbecco, 1972; Keller & Wendel, 1974).

The model most closely consistent with present findings is that the tertiary structure of viral DNA is generated by a single protein with an average lifetime of approximately 1 1/2 hours which functions by attachment to newly-synthesized DNA during or immediately after closure. This is suggested by the fact that the formation of component Ic requires prerequisite DNA replication, but upon reversal of inhibition of protein synthesis, the Ic molecules may return to the normal superhelix density of form I by a process independent of replication; moreover, restoration

of the form I conformation proceeds with the same kinetics as diversion of the synthesis of form I to Ic during cycloheximide inhibition.

Much work is still required before it is possible to elucidate completely the involvement of concurrent protein synthesis and how this couples with viral and cellular DNA replication. The extent of participation of histone is of particular interest. Furthermore, research in the future can be centred around one of the following themes:

The development of an *in vitro* assay for the protein(s) responsible for the generation of proper tertiary structure in polyoma DNA based on the introduction of superhelical turns into purified component Ic would be a logical sequel to this work. Following the establishment of such an assay system, the isolation, purification and study of this factor will then become possible. The initiation protein can be similarly handled.

APPENDIX

I. Chemicals

Unless noted specially, all general chemicals used were either analytical grade obtained from British Drug Houses or Baker Chemicals; otherwise, reagent grade (A.C.S. certified) obtained from Fisher Scientific Co. or Shawinigan Chemicals were used.

Cesium chloride used for isopycnic density gradients was of optical grade, purchased from the Harshaw Chemical Co., of the Kewanee Oil Company, Solon, Ohio, U.S.A.

Sucrose used for velocity sedimentation analyses were of density gradient grade, crystalline, (ribonuclease free) from Schwarz/Mann of the Becton, Dickinson and Company, Orangeburg, New York, U.S.A.

Propidium iodide A grade 1 1/2 hydrate was purchased from CalBiochem, San Diego, California (No. 537059).

Special chemicals ordered from Sigma Chemical Company, St. Louis, Missouri, U.S.A. include the following:

Bovine Albumin (Fraction V) powder-96-99% Albumin	A-4503
Caffeine, Anhydrous	C-0750

Cytosine-1-β-D-Arabinofuranosyl HCl (Cytosine Arabinoside Hydrochloride)	C-3631
Cycloheximide (3[2(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide	C-6255
Deoxyribonuclease I (1 x crystallized & lyophilized) (Bovine pancreas) 2700 Kunitz* units/mg	DN-C
5 Fluorodeoxyuridine (crystalline)	F-0503
Hydroxyurea	H-8627
N-Lauroyl Sarcosine (Sodium salt)	L-5125

II. Radioactive isotopes

(i) ^{14}C-thymidine was purchased from Amersham/Searle (code CFA.219) in the form of [2-^{14}C] thymidine with a specific activity of 61 mCi/m mole.

(ii) ^{3}H-thymidine was purchased from New England Nuclear (code NET-027Z) in the form of thymidine [methyl-^{3}H] with a specific activity of 40-60 Ci/m mole.

(iii) ^{3}H-amino acid mixture was purchased from New England Nuclear (code NET-250) in the form of L-amino acid mixture [^{3}H(G)] with a concentration of 1 mCi/ml 0.1N HCl and 15 purified L-amino acids in the mixture.

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*1 Kunitz= Δ O.D.$_{260}$ 0.001/min/ml. at pH 5 at 25°C with DNA as substrate,

III. Toluene base liquid scintillation fluid. (Arnold, 1963)

PPO (2,5-diphenyloxazole) (Amersham/Searle)	6g
POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene. (Amersham/Searle)	0.1g

q.s. 1 litre with sulfur free toluene from BDH chemicals (A.C.S. specification)

IV. Solutions for tissue culture

Most of the solutions and chemicals required for tissue culture was purchased from Grand Island Biological Company (GIBCO), Grand Island, New York; these include the following:-

Foetal calf serum (heat inactivated)	614HI
Fungizone (Amphotericin B 250 mcg/ml)	529
Modified McCoy 5A Medium (with L-Glutamine but without sodium bicarbonate*)	H-15
Penicillin-Streptomycin 10,000 u & 10,000 mg/ml	514
Trypsin 2.5%** (1:250) lyophilized	509L

* 0.25% (w/v) $NaHCO_3$ was added before filter sterilization.

**Trypsin 2.5% was used together with Versene 0.2 g/l; glucose 0.2 g/l & phenol red 0.02 g/l q.s. 1 l with calcium magnesium free PBS to form the Trypsin-EDTA Solution.

V. Buffers & Solutions

PDI buffer — pH 8.6 (Bourgaux, Bourgaux-Ramoisy, 1972 a)

EDTA	0.02M
Tris.HCl	0.02M

Buffer A — pH 8.1 (Kiger & Sinsheimer, 1969)

NaCl	0.3M
EDTA	0.001M
Tris	0.01M

Neutral EDTA buffer — pH 7.4 (Penman, Vesco, Penman, 1968)

NaCl	0.1M
EDTA	0.001M
Tris	0.01M

SA buffer

NaCl	0.5M
NaOH	0.25N
EDTA	0.001M

LA buffer

NaCl	0.7M
NaOH	0.3N
Tris	0.01M

Neutral LA buffer (pH 7.4)

NaCl	1.0M
EDTA	0.001M
Tris	0.01M

Standard Saline Citrate (SSC) (Marmur, 1961)

NaCl	0.15M
Sodium citrate	0.015M
pH	7.8

Phosphate Buffered Saline (PBS) (Dulbecco & Vogt, 1954)

*$MgCl_2.6H_2O$	0.1g
*$CaCl_2$ (anhyd.)	0.1g
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g

q.s. double glass distilled water to 1 litre.

*Omit for "calcium-magnesium free" solutions.

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